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Resveratrol Sensitizes AML Cells to HDAC Inhibitors via ROS-Mediated Activation of the Extrinsic Apoptotic Pathway

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Running Title: resveratrol increases HDAC inhibitor anti-AML activity

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Abbreviations: HADCI, histone deacetylase inhibitor; vorinostat, suberoylanilide hydroxamic acid (SAHA); LBH-589, panobinostat; AML, acute myelogenous leukemia; FLT3-ITD, internal tandem duplications of FLT3; shRNA, short pairpin RNA; shNC, negative control scrambled shRNA; MnTBAP, Mn(III)tetrakis(4-benzoic acid)porphyrin chloride, a cell-permeable SOD mimetic and peroxynitrite scavenger; SOD, superoxide dismutase; ROS, reactive oxygen species; DR5, death receptor 5; DN caspase-8, dominant-negative caspase-8; NF-κB, nuclear factor-kappaB; Sirt1, sirtuin (silent mating type information regulation 2 homolog) 1; IKK, IkappaB kinase; PARP, poly ADP (adenosine diphosphate)-ribose polymerase; 7-AAD, 7-

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Aminoactinomycin D; $DiOC_6$, 3,3'-dihexyloxacarbocyanine iodide; H_2DCFDA , cell-permeant 2',7'-dichlorodihydrofluorescein diacetate; EdU, 5-ethynyl-2'-deoxyuridine; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum.

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Abstract

HDAC inhibitors (HDACIs) activate the pro-survival NF-κB pathway by hyperacetylating RelA/p65, whereas the chemopreventive agent resveratrol inhibits NF-κB by activating the class III HDAC Sirt1. Consequently, interactions between resveratrol and pan-HDACIs (vorinostat and LBH-589) were examined in human acute myelogenous leukemia (AML) cells. Pharmacologically achievable resveratrol concentrations (e.g., 25-50 μM) synergistically potentiated HDACI lethality in AML cell lines and primary AML blasts. Resveratrol antagonized RelA acetylation and NF-κB activation in HDACI-treated cells. However, shRNA Sirt1 knockdown failed to modify HDACI sensitivity, suggesting that factors other than or in addition to Sirt1 activation contribute to resveratrol/HDACI interactions. These interactions were associated with DR5 up-regulation and caspase-8 activation, while cells expressing dominant-negative caspase-8 were substantially protected from HDACI/resveratrol, arguing for a significant functional role for the extrinsic apoptotic pathway in lethality. Resveratrol/HDACI co-exposure induced sustained ROS generation accompanied by increased DNA DSBs, reflected by γH2A.X and comet assays. Significantly, the free radical scavenger MnTBAP blocked ROS generation, DR5 up-regulation, caspase-8 activation, DNA damage, and apoptosis, indicating a primary role for oxidative injury in lethality. Analyses of cell cycle and EdU incorporation by flow cytometry revealed that resveratrol induced S-phase accumulation, an effect abrogated by HDACI co-administration, suggesting that cells undergoing DNA synthesis may be particularly vulnerable to HDACI lethality. Collectively, these findings indicate that resveratrol interacts synergistically with HDACIs in AML cells via multiple ROS-dependent actions, including death receptor up-regulation, extrinsic apoptotic pathway activation, and DNA damage induction. They also raise the possibility that S-phase cells may be particularly susceptible to these actions.

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Introduction

Histone deacetylase inhibitors (HDACIs) represent a class of epigenetic agents that regulate gene expression by modifying chromatin structure. HDACIs promote histone acetylation, leading to a more relaxed configuration conducive to the transcription of genes implicated in differentiation and cell death (Bolden et al., 2006). However, HDACIs also kill transformed cells through alternative mechanisms, including induction of oxidative injury (Ruefli et al., 2001), interference with DNA repair machinery (Subramanian et al., 2005), and up-regulation of death receptors (Nebbioso et al., 2005) among others. Notably, the pan-HDACI vorinostat has been approved for the treatment of cutaneous T-cell lymphoma (Grant et al., 2007), and initial suggestions of HDACI activity in acute myelogenous leukemia (AML) have been reported (Garcia-Manero et al., 2008).

HDACs are sub-divided into four groups: Class I (HDAC1-3 and 8), analogous to yeast RPD; Class II (HDAC4-7, 9, and 10), analogous to yeast HDAI; the NAD^+-dependent sirtuins (Sirt1-7); and Class IV (HDAC11) (Glozak and Seto, 2007). Sirtuins have been implicated in the regulation of tumor initiation, progression, and chemoresistance, and consequently, agents that modify sirtuin activity are currently a subject of interest in cancer therapy (Liu et al., 2009). Resveratrol is a naturally occurring polyphenolic compound extracted from grapes, and clinical trials are underway to explore its potential in patients with cardiovascular diseases or diabetes (Baur and Sinclair, 2006). It has been associated with minimal toxicity and plasma levels > 300 μM are achievable and well-tolerated in humans (Howells et al., 2011). In preclinical studies, resveratrol exhibits activity against various malignant cell types including AML (Tsan et al., 2002) through diverse mechanisms e.g., inhibition of IKK and NF-κB (Holmes-McNary and Baldwin, Jr., 2000), induction of oxidative injury (Low et al., 2010), or autophagy (Puissant et al., 2010). Interestingly, resveratrol has been shown to act as a Sirt1 agonist (Milne et al., 2007), although evidence has emerged indicating that this may involve indirect actions (Pacholec et al., 2010).

In addition to histones, HDACIs promote the acetylation of diverse non-histone proteins, including transcription factors such as NF-κB (Glozak et al., 2005). In previous studies, we reported that inhibitors of the NF-κB signaling pathway, including IKK or proteasome inhibitors, markedly increased the activity of HDACIs against myeloid leukemia cells (Dai et al., 2005; Dai

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et al., 2011b). Among other actions, these agents potently block RelA acetylation, which plays an important role in DNA binding and transactivation (Dai et al., 2005). Moreover, it is known that like class I HDACs (e.g., HDAC3), the class III HDAC Sirt1 also deacetylates RelA and inactivates NF-κB (Chen et al., 2005). However, pan-HDACIs such as LBH-589 and vorinostat fail to target Class III HDACs (Xu et al., 2007). Furthermore, sirtuin agonists, by antagonizing RelA acetylation, have been shown to inhibit NF-κB function (Dai et al., 2005; Yeung et al., 2004). Collectively, these observations raised the possibility that resveratrol might sensitize leukemia cells to HDACIs. To address this question, we have examined interactions between resveratrol and two clinically-relevant pan-HDACIs (vorinostat and LBH-589/panobinostat) in human myeloid leukemia cells. Here we report that resveratrol synergistically potentiates HDACI activity against myeloid leukemia cells in association with the ROS-dependent activation of the extrinsic apoptotic pathway.

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Materials and Methods

Cells and Cell Culture

U937 and MV-4-11 (bearing FLT3-ITD) human leukemia cells were obtained from American Type Culture Collection (ATCC, Manassas, VA), and maintained in RPMI1640 medium containing 10% FBS as previously described (Maggio et al., 2004). U937 cells were stably transfected with dominant-negative (DN) caspase-8 or their empty vector counterparts as previously described (Rosato et al., 2007).

Bone marrow (BM) or peripheral blood (PB) samples (> 65% blasts) were obtained with informed consent, in accordance with the Declaration of Helsinki, from patients with histologically documented AML undergoing routine diagnostic procedures with IRB approval (VCU IRB #HM 12517). Mononuclear cells were isolated by centrifugation at 400g for 30 min over Histopaque-1077 (Sigma Diagnostics, St. Louis, MO). Cell viability was regularly > 95%; all samples consisted of > 70% blasts. All experiments were performed at a density of 1 x 10^6 cells/ml as described previously (Dai et al., 2011b).

Drugs and Chemicals

Resveratrol (3,4′,5-Trihydroxy-*trans*-stilbene) and sodium azide were purchased from Sigma-Aldrich (St. Louis, MO). Vorinostat (Zolinza, suberoylanilide hydroxamic acid [SAHA]) and LBH-589 (panobinostat) were provided by Merck (Whitehouse Station, N.J.) and Novartis Pharmaceuticals Inc. (East Hanover, NJ) respectively. The SOD mimic MnTBAP (Mn[III] tetrakis[4-benzoic acid] porphyrin chloride) was obtained from Calbiochem (Billerica, CA). The selective NADPH oxidase (NOX1) inhibitor ML171 (2-acetylphenothiazine) was purchased from Millipore (Billerica, MA). Reagents were formulated in DMSO and stored at -20^{o}C. Sodium azide was dissolved in sterile PBS before use. Stock solutions were subsequently diluted with serum-free RPMI medium prior to use to ensure that the final concentration of DMSO did not exceed 0.02%.

Experimental Format

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Logarithmically growing cells ($2.5\text{-}4.0 \times 10^5$ cells/ml) were exposed to various concentrations of HDACIs in the presence or absence of resveratrol for indicated intervals, generally 24 – 48 hrs, after which cells were processed and assayed.

RNA Interference

SureSilencing shRNA plasmids (neomycin resistance) were purchased from SABioscience (Frederick, MD), which includes shSirt1 (human sirtuin 1; Gene ID 23411) and negative control shRNA (shNC). U937 cells were stably transfected with these constructs by using the Amaxa Nucleofector device with Cell Line Specific Nucleofector Kit C (Amaxa GmbH, Cologne, Germany) as per the manufacturer's instructions, and clones with down-regulated expression of Sirt1 were selected with 400μg/ml G418.

Assessment of Cell Death and Mitochondrial Membrane Potential

Cells were double-stained with a solution containing 25 μM 7AAD and 40 nM $DiOC_6$ for 20 min at 37°C, and then analyzed by BD Biosciences FACScan flow cytometry as described earlier (Maggio et al., 2004)**.** In some cases, apoptosis was evaluated by annexin V/propidium iodide (PI) (BD PharMingen, Franklin Lakes, NJ) staining for 30 min at room temperature and flow cytometry as described (Rosato et al., 2010).

Measurement of ROS Production

Cells were treated with 20 μM H_2DCFDA (Molecular Probes, Eugene, OR) for 30 min at 37°C, and then analyzed by flow cytometry as described previously (Rosato et al., 2010).

Comet Assay

Single-cell gel electrophoresis assays were performed to assess both single- and double-stranded DNA breaks in cells using a Comet Assay Kit (Trevigen, Gaithersburg, MD) as per the manufacturer's instructions.

Western Blot Analysis

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Whole cell-pellets were washed in PBS, and lysed with loading buffer (Invitrogen, Carlsbad, CA) as previously described (Maggio et al., 2004). Alternatively, S-100 cytosolic fractions were prepared as described previously (Dai et al., 2005). 30 μg of total protein for each condition were separated by 4-12% Bis-Tris NuPAge precast gel system (Invitrogen, Grand Island, NY) and electro-blotted to nitrocellulose. After incubation with the corresponding primary and secondary antibodies, blots were developed by enhanced chemiluminesence (New England Nuclear; Boston, MA). Primary antibodies were as follows: caspase-8 (ENZO Life Sciences, Plymouth Meeting, PA); caspase-3 (BD Transduction Lab, San Diego, CA); caspase-9, DR5, cytochrome c, p65 (BD Biosciences, San Jose, CA); acetyl-NF-κB p65 (Lys 310), cleaved caspase-3 and -9 (Cell Signaling Technology, Beverly, MA); PARP (BioMol, Plymouth Meeting, PA); Sirt1 (Santa Cruz, Santa Cruz, CA); γH2A.X (S139, Millipore, Billerica, MA); β-actin (Sigma) and α-tubulin (Oncogene Inc.). Secondary antibodies conjugated to horseradish peroxidase were from Kirkegaard and Perry Laboratories, Inc. (Gaithersburg, MD).

Immunoprecipitation

RelA acetylation was evaluated by immunoprecipitation/Western blot analysis as described previously (Dai et al., 2005). Briefly, 200 μg proteins per condition were incubated under continuous shaking with 1 μg NF-κB p65 antibody (mouse monoclonal, Santa Cruz Biotech) overnight at 4^{o}C. 20 μl/condition of Dynabeads (Goat anti-mouse IgG, Dynal, Oslo, Norway) were added and incubated for an additional 4 hr. After washing three times with RIPA buffer, the bead-bound protein was eluted by vortexing and boiling in 20 μl 1x sample buffer. The samples were separated by SDS-PAGE and subjected to Western blot analysis as described above. Acetylated-lysine antibodies (Millipore, Billerica, MA) were employed as primary antibodies.

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ELISA-based NF-κB p65 activity analysis

Nuclear protein was extracted using Nuclear Extract Kit (Active Motif, Carlsbad, CA). RelA/p65-specific DNA binding activity in nuclear extracts was measured using Nuclear Extract and TransAM^{TM} NF-κB p65 Chemi Kits (Active Motif) as we recently described (Rosato et al., 2010).

Cell Cycle Analysis

Cell cycle analysis of DNA content by propidium iodide (PI) staining was performed by flow cytometry using Modfit LT2.0 software (Topsham, ME) as described previously (Pei et al., 2011).

DNA Synthesis (S Phase) Analysis

Click-iT™ EdU CellCycle AF488-red (7-AAD) Assay Kit (Invitrogen) was used, as per the manufacturer's instructions, to determine S phase via incorporation of the thymidine analogue 5-ethynyl 2-deoxyuridine (EdU) into genomic DNA during DNA synthesis by flow cytometry. Alternatively, cells were double-stained with EdU-AF488 and Annexin V-APC, after which flow cytometry was performed to determine percentage of apoptosis (Annexin V^+) in the S phase (EdU^+) population.

Statistical Analysis

For analyses of cell death, ROS production, and NF-κB activity, values represent the means ± SD for at least three separate experiments performed in triplicate. One-way ANOVA with Tukey-Kramer Multiple Comparisons Test and two-tailed Student t test were performed. Analysis of synergism was performed according to Median Dose Effect analysis using the software program Calcusyn (Biosoft, Ferguson, MO).

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Results

Resveratrol synergistically interacts with HDACIs in human myeloid leukemia cells

Co-administration (24 hr) of a marginally toxic concentration (50 μM) of resveratrol significantly increased the lethality of minimally toxic concentrations (10-20 nM) of LBH-589 in U937 cells (**Fig. 1A**). This event was observed at resveratrol concentrations as low as 10 μM (**Fig. 1B**). Time course analysis revealed increases in lethality for the resveratrol/LBH-589 regimen first discernible at 16 hr, which became more pronounced over the ensuing 32 hr (**Fig. 1C**). Analogous results were obtained when another pan-HDACI (vorinostat; 1.5 μM) was employed in combination with resveratrol (**Supplemental Fig. S1A).** Similar interactions were observed in MV4-11 cells, an AML line bearing the FLT3-ITD mutation, employing relatively lower concentrations (e.g., 7.5 nM) of LBH-589 (**Fig. 1D**). Median Dose Effect analysis in U937 cells exposed to a range of resveratrol and LBH-589 concentrations at the indicated fixed ratio yielded combination index values (CI = 0.243 – 0.782, Annexin V/PI analysis) less than 1.0, indicating synergistic interactions (**Fig. 1E**). Synergism between resveratrol and vorinostat was also observed (CI = 0.614– 0.976, Annexin V/PI analysis; **Supplemental Fig. S1B**). Consistent with these findings, resveratrol interacted synergistically with LBH-589 or vorinostat in triggering loss of mitochondrial membrane potential ($\Delta\psi m$, determined by $DiOC_6$ uptake; **Supplemental Fig. S1C**). Notably, primary AML blasts displayed no or minimal toxicity when exposed (24 hr) to 50 μM resveratrol, 10 nM LBH-589 or 1.0 μM vorinostat alone, but combined treatments resulted in a sharp increase in cell death (e.g., **Fig. 1F**).

Co-administration of HDACIs with resveratrol leads to enhanced DNA damage, mitochondrial injury, and caspase-3, -9, and -8 activation

Whereas resveratrol or HDACIs (LBH-589 or vorinostat) administered individually for 16 hr or longer minimally induced γH2A.X, an indicator of DNA double-strand breaks (Rosato et al., 2010), combined treatments sharply increased γH2A.X expression in U937 and MV-4-11 cells (**Fig. 2A**). Moreover, after drug treatment (24 hrs), single- and double-stranded DNA breaks were analyzed by comet assays in U937 cells. In this assay, denatured, broken DNA fragments migrate out of the cell under the influence of an electric field, producing a comet tail, whereas undamaged DNA migrates more slowly and remains within the confines of the nucleus

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(Dai et al., 2008). As shown in **Fig. 2C**, co-administration of resveratrol with either LBH-589 or vorinostat resulted in a clear increase in the number of comet-positive cells compared to treatment with the agents individually. Notably, the appearance of DNA comet tails in cells co-exposed to resveratrol with LBH-589 or vorinostat occurred substantially before the induction of massive apoptosis i.e., 8 hr (**Fig. 2C**) vs 24 hr (**Fig. 1C**). Furthermore, co-administration of resveratrol with LBH-589 or vorinostat also resulted in early (4 – 8 hr) and marked increases in release of mitochondrial cytochrome c into the cytosol when compared to individual treatment (**Fig. 2B**). Lastly, co-exposure to either resveratrol/LBH-589 or /vorinostat led to clearly increased cleavage/activation of caspase-3, caspase-9, and particularly caspase-8, accompanied by marked PARP degradation in U937 (**Fig. 2B**) and MV-4-11 cells (**Fig. 2C**).

Resveratrol blocks HDACI-mediated RelA acetylation and NF-κB *activation*

Previous studies have shown that interference with RelA/p65 acetylation and NF-κB activation e.g., by IKK inhibitors, dramatically increases HDACI lethality in AML cells,(Dai et al., 2005) raising the possibility that a Sirt1 agonist such as resveratrol might act similarly by promoting Sirt1-mediated RelA deacetylation. To address this question, the effects of resveratrol on RelA/p65 acetylation and NF-κB were examined in U937 cells exposed to HDACIs. As shown in **Fig. 3A,** ELISA-based NF-κB activity analysis of nuclear extracts showed that exposure to both LBH-589 and vorinostat induced p65-specific NF-κB activation as previously reported (Dai et al., 2005), while this event was significantly blocked by resveratrol ($P < 0.001$ in each case). Western blot analysis of whole-cell lysates demonstrated that co-administration of resveratrol with either LBH-589 or vorinostat clearly diminished K310 acetylation of p65 (**Fig. 3B**). Moreover, immunoprecipitation analysis also revealed that resveratrol co-administration substantially diminished HDACI-induced p65 acetylation (**Fig. 3C**). Together, these findings indicate that resveratrol attenuates RelA/p65 acetylation and NF-κB activation in HDACI-treated AML cells, similar to effects observed with other agents that directly target the NF-κB signaling pathway such as IKK and proteasome inhibitors (Dai et al., 2005;Dai et al., 2011b).

Knock-down of Sirt1 fails to attenuate HDACI lethality

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To investigate the functional significance of perturbations in Sirt1 in regulating HDACI lethality, U937 cells were stably transfected with constructs encoding shRNA specifically targeting human Sirt1 (shSirt1) or a scrambled sequence as a negative control (shNC). Two shSirt1 clones (designated 36 and 45) were isolated that displayed sharp reductions in Sirt1 expression compared to shNC cells (**Fig. 3D**; left panel). However, contrary to expectations that Sirt1 knock-down would exert effects opposite to those of the Sirt1 agonist resveratrol, both shSirt1 clones appeared slightly more sensitive, rather than resistant, to LBH-589 or vorinostat compared to controls, although differences did not achieve statistical significance (**Fig. 3D**, right panel; $P > 0.05$). This finding argues that mechanisms other than or in addition to Sirt1 activation by resveratrol contribute to potentiation of HDACI anti-leukemic activity by this agent.

Resveratrol/HDACI activity proceeds through an ROS-dependent process

Earlier studies have shown that both resveratrol (Schilder et al., 2009; Low et al., 2010) and HDACIs (Ruefli et al., 2001; Rosato et al., 2010) can trigger cell death through an oxidative-injury mediated process. Consequently, the effects of resveratrol ± HDACIs on generation of reactive oxygen species (ROS) were examined. Exposure of U937 cells to LBH-589 or vorinostat alone triggered modest increases in ROS which declined slightly after 24-hr exposure (**Fig. 4A**). In contrast, resveratrol alone induced a sharp increase in ROS which persisted over the 24-hr exposure interval, while co-administration of HDACIs did not increase ROS accumulation further (**Fig. 4A**). Significantly, ROS generation in cells exposed to resveratrol ± HDACIs was largely abrogated by the ROS scavenger MnTBAP (**Fig. 4B**), leading to substantial protection from resveratrol/HDACI-induced cell death (**Fig 4C**) as well as loss of mitochondrial membrane potential (**Supplemental Fig. S2A**) and cleavage of caspase-3 and PARP (**Supplemental Fig. S2B**). Moreover, it is known that NADPH oxidases (NOXs) share the capacity to transport electrons across the plasma membrane and reduce oxygen to superoxide, thereby generating downstream ROS (Gianni et al., 2010). In this context, a selective NOX1 inhibitor ML171 (Gianni et al., 2010) was employed to assess the functional role of NOXs in the lethality of the resveratrol/HDACI regimen. As shown in **Fig. 4D**, 1 hr pre-treatment with ML171 significantly prevented apoptosis induced by co-administration of resveratrol with LBH-589 ($P = 0.0079$ vs without ML171) or vorinostat ($P = 0.0419$) in U937 cells. Furthermore, catalases efficiently

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decompose H_2O_2 derived from superoxide (O_2^-), a reaction catalyzed by superoxide dismutase (SOD), in cells (Mesquita et al., 2010). Therefore, two catalase inhibitors were used to analyze the functional role of catalase in cell death induced by resveratrol +/- HDACIs. It was found that the specific catalase inhibitor 3-AT (3-amino-1,2,4-triazole) (Mesquita et al., 2010) failed to attenuate the lethality of the resveratrol regimen (data not shown). Interestingly, 1 hr pre-treatment with sodium azide, a known catalase inhibitor and a donor of nitric oxide (NO), which has previously been reported to prevent apoptosis via the extrinsic pathway (Kim et al., 1997), in the presence of catalase and H_2O_2 (Ogino et al., 2001), substantially prevented apoptosis induced by co-exposure to resveratrol and LBH-589 ($P = 0.0110$ vs without azide) or vorinostat ($P = 0.0151$) in U937 cells (**Fig. 4E**).

The ROS scavenger MnTBAP blocks DR5 up-regulation, caspase-8 cleavage, and DNA damage in cells co-exposed to resveratrol/HDACI

As shown in **Fig. 2D**, caspase-8 exhibited pronounced cleavage in U937 cells after co-exposure to resveratrol/HDACI, indicating activation of the extrinsic apoptotic cascade. In this context, resveratrol by itself induced expression of the death receptor DR5 (**Fig. 5A**), as recently reported in DLBCL cells (Hussain et al., 2011). Notably, co-administration of LBH-589 clearly enhanced resveratrol-mediated DR5 up-regulation (**Fig. 5A**), accompanied by increased caspase-8 cleavage (**Fig. 5B**) and γH2A.X expression (**Fig. 5A**). However, in the presence of MnTBAP, DR5 induction as well as caspase-8 cleavage/activation were entirely abrogated, and γH2A.X expression (DNA damage) largely prevented (**Fig. 5A** and **5B).** Together, these findings argue that induction of ROS acts upstream of other lethal events (e.g., activation of the extrinsic death pathway and DNA damage), and thus plays a primary functional role in the anti-leukemic activity of the resveratrol/HDACI regimen.

Evidence of a functional role for the extrinsic pathway in resveratrol/HDACI lethality

To assess the functional significance of activation of the extrinsic apoptotic pathway in this setting more definitively, U937 cells expressing dominant-negative (DN) caspase-8 were employed (Rosato et al., 2007). Significantly, cleavage/activation of both caspases-8 and -3 induced by resveratrol/LBH-589 or /vorinostat were dramatically diminished in DN caspase-8

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cells compared to their empty-vector counterparts (**Fig. 5C**). Consistent with these results, resveratrol/HDACI lethality was significantly attenuated in DN caspase-8 cells ($P < 0.02$ or 0.05 vs. empty-vector control, **Fig. 5D**). Collectively, these findings indicate that ROS-dependent activation of the extrinsic pathway plays a significant functional role in the anti-leukemic activity of this regimen.

Resveratrol induces S-phase accumulation and sensitizes leukemic cells to HDACIs

Resveratrol has been shown to induce S-phase arrest in various tumor cell types, including leukemia cells (Bernhard et al., 2000). Consequently, the effects of resveratrol ± HDACIs on cell cycle progression was examined. As reported previously (Bernhard et al., 2000), exposure to resveratrol induced a marked increase in S-phase cells in a time-dependent manner (**Fig. 6A**). In contrast, exposure to HDACIs (e.g., vorinostat) resulted in a modest increase of cells in G_0G_1 phase, accompanied by a slight decline in the S-phase population, but little increase in the hypodiploid population. However, co-administration of vorinostat virtually eliminated the S-phase accumulation of cells induced by resveratrol, followed by a clear increase in the sub-diploid (sub-G_1) population (**Fig. 6A** and **Supplemental Fig. S2C**). In accord with these findings, flow cytometric analysis of EdU incorporation, which reflects DNA-synthesis, demonstrated a pronounced increase in EdU-positive S-phase cells following exposure to resveratrol (e.g., 72% vs. 46% in untreated cells), whereas co-administration of vorinostat with resveratrol led to the virtual disappearance of EdU-positive cells (e.g., to 4%; **Fig. 6B**). Moreover, double-staining with EdU-AF488 and Annexin V-APC demonstrated that co-administration of resveratrol with either LBH-589 or vorinostat induced apoptosis in both the EdU-positive (e.g., 58% or 44% for resveratrol + LBH-589 or vorinostat, respectively, vs 15% for resveratrol alone; **Fig. 6C**), as well as the EdU-negative population. Together, these findings raise the possibility that resveratrol arrests leukemia cells in S-phase, and that such cells may be particularly susceptible to HDACI-mediated lethality.

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Discussion

Although resveratrol has traditionally been viewed as a chemopreventive agent (Baur and Sinclair, 2006), recent studies have highlighted its capacity to induce cell death in neoplastic cells, including leukemia cells (Puissant et al., 2010). Like numerous natural products, the mechanisms by which resveratrol triggers transformed cell death are likely to be multi-factorial (Athar et al., 2009). Resveratrol acts as an agonist of Sirt1 (Park et al., 2012), a member of the class III HDAC subfamily that pan-HDACIs fail to target (Xu et al., 2007), and exerts inhibitory effects on NF-κB, which contributes its anti-cancer activity (Dai et al., 2005;Yeung et al., 2004). Resveratrol has also been shown to prevent NF-κB activation and NF-κB-dependent gene expression through its inhibitory effects on IKKs (Holmes-McNary and Baldwin, Jr., 2000). In transformed cells, HDACIs activate NF-κB via RelA (S536) phosphorylation (Dai et al., 2011a) through an ATM/NEMO-dependent mechanism (Rosato et al., 2010). Moreover, HDACIs induce RelA/p65 acetylation, which prevents nuclear export, while promoting DNA binding and transactivation (Chen et al., 2002). Consequently, these events lead to up-regulation of several anti-apoptotic and anti-oxidant proteins, which diminish HDACI lethality, as well as activation of the stress-related JNK pathway (Dai et al., 2005). In this context, agents that prevent NF-κB activation, such as IKK (Dai et al., 2010) or proteasome inhibitors (Dai et al., 2011b), have been shown to markedly increase HDACI lethality in malignant human hematopoietic cells, including leukemia cells. Therefore, we hypothesized that resveratrol, like NF-κB inhibitory agents, might also enhance HDACI-mediated anti-leukemia. Consistent with this hypothesis, resveratrol clearly diminished p65 acetylation (e.g., K310) and NF-κB activation in HDACI-treated leukemia cells, accompanied by a sharp increase in cell death.

The observation that most HDACIs do not target class III HDACs, including Sirt1, provides a rationale for combining Sirt1 agonists like resveratrol with HDACIs in cancer treatment. While the Sirt1 agonist activity of resveratrol may proceed through an indirect mechanism (Pacholec et al., 2010), it has nevertheless been demonstrated that this agent exerts its effects by activating Sirt1, which negatively regulates p65 acetylation, an event essential for sustained NF-κB activation (Chen et al., 2001). Therefore, it was anticipated that if Sirt1 activation by resveratrol (Milne et al., 2007) was responsible for potentiating HDACI lethality, then knock-down of Sirt1 would be expected to attenuate HDACI-mediated cell death, contrary

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to the effects of a Sirt1 agonist. Unexpectedly, Sirt1 knock-down failed to protect leukemia cells from HDACI-mediated lethality, raising several possibilities. First, other resveratrol actions (e.g., pro-oxidant activity, disruption of cell cycle, etc.) (Athar et al., 2009) may be primarily responsible for potentiation of HDACI lethality. Alternatively, aside from deacetylation of p65, additional Sirt1 actions may play predominantly cytoprotective roles (Chen et al., 2009). For example, Sirt1 is known to mediate deacetylation of numerous non-histone substrates (e.g., FOXOs, p53, NF-κB, PPARγ, and PGC-1α, etc.). In support of this notion, pharmacologic Sirt1 antagonists have recently been shown to promote CML stem cell death by increasing acetylation and transcriptional activity of p53.(Li et al., 2012)

While the chemopreventive actions of resveratrol may stem from its anti-oxidant properties (de la Lastra and Villegas, 2007), resveratrol has been shown to be a potent inducer of tumor cell oxidative injury (Chandra, 2009). In leukemia cells, oxidative injury has also been shown to represent an important mechanism of HDACI lethality (Petruccelli et al., 2011;Ruefli et al., 2001;Rosato et al., 2003). The observation that ROS generation played a critical functional role in actions of this combination regimen is not surprising. Notably, resveratrol administered alone induced a pronounced and sustained increase in ROS, but this was not accompanied by marked DNA damage (expression of γH2A.X or formation of comet tails) or cell death. However, while HDACIs did not further increase ROS production in resveratrol-treated cells, co-administration sharply increased DNA damage and cell death. Given evidence that HDACIs interfere with DNA repair processes i.e., by hyperacetylating DNA repair proteins such as Ku70 (Subramanian et al., 2005) or down-regulating others (e.g., Rad50 or MRE11) (Lee et al., 2010), it is tempting to speculate that HDACIs amplify the lethal consequences of resveratrol-mediated oxidative injury and resulting DNA damage (Subramanian et al., 2005).

Co-administration of resveratrol and HDACIs triggered activation of caspases, particularly caspase-8, indicating activation of the extrinsic apoptotic cascade (Scaffidi et al., 1998). In this context, HDACIs are known to up-regulate death receptors in human leukemia cells (Insinga et al., 2005), and very recently, resveratrol has also been shown to up-regulate death receptors, including DR5, in lymphoma cells (Hussain et al., 2011). Consistent with these observations, co-exposure of leukemia cells to resveratrol and HDACIs increased DR5 expression compared to the effects of each agent administrated individually. This finding

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provides a potential explanation for the marked activation of the extrinsic pathway by the resveratrol/HDACI regimen. Importantly, blockade of the extrinsic pathway by dominant-negative caspase-8 substantially diminished resveratrol/HDACI lethality, demonstrating a significant functional role for activation of this pathway in the anti-leukemia activity of this strategy. Notably, both up-regulation of DR5 and activation of the extrinsic pathway were essentially abrogated by the ROS scavenger MnTBAP. Of note, similar phenomena have also been observed in human lymphoma (DLBCL) cells exposed to resveratrol alone (Hussain et al., 2011). Together, these findings argue that ROS-dependent induction of death receptors (e.g., DR5) and resulting activation of the extrinsic apoptotic pathway represents an important mechanism underlying anti-leukemic synergism between these agents. Finally, while the ability of ML171 to protect cells from resveratrol/HDACI lethality implicates NOXs in this phenomenon, additional studies will be required to identify the source(s) of the ROS generated more definitively.

Resveratrol has been reported to synchronize cells in S-phase in association with inhibition of cdc2 (Tyagi et al., 2005) or NF-κB (Estrov et al., 2003). The results of the present study demonstrated that resveratrol exposure sharply increased the S phase population and the percentage of EdU-positive cells, a specific S-phase marker reflecting DNA synthesis (Kramer and Wesierska-Gadek, 2009), in a time-dependent manner. Notably, co-administration of HDACIs prevented resveratrol-induced S-phase accumulation and DNA synthesis in association with a marked increase in the sub-diploid fraction, reflecting apoptosis. One possible explanation for these findings is that cells exposed to resveratrol accumulated in S-phase, which is characterized by active DNA synthesis, and that such cells may be particularly sensitive to HDACIs, as observed in the case of the classic S-phase synchronizer hydroxyurea (Kramer et al., 2008). Previous findings that abrogation of G_0G_1 arrest by blocking induction of the endogenous CDK inhibitor $p21^{CIP1}$ markedly sensitizes leukemia cells to HDACIs (Rosato et al., 2002) is compatible with this notion. Alternatively, the cytoprotective effects of HDACI-mediated NF-κB activation, and particularly its antioxidant actions due to induction of proteins such as SOD2 (Dai et al., 2005) may be critical for the survival of S-phase cells, which are known to be particularly vulnerable to oxidative injury (Ge et al., 2006). Finally, HDACIs kill transformed cells through diverse mechanisms, including up-regulation of pro-apoptotic proteins such as Bim

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(Zhao et al., 2005), induction of death receptors (Nebbioso et al., 2005), and promotion of DNA damage (Lee et al., 2010), among numerous others. Additional studies will be required to define the relative contributions of these actions to HDACI lethality toward S-phase cells.

In summary, the present studies demonstrate that resveratrol, administered at pharmacologically achievable concentrations (Howells et al., 2011), significantly increases HDACI lethality in human AML cells through the oxidative injury-mediated activation of the extrinsic apoptotic pathway. The results also indicate that while resveratrol does block HDACI-mediated NF-κB acetylation and activation, events previously implicated in potentiation of HDACI-mediated leukemic cell death (Dai et al., 2005; Rosato et al., 2010), actions other than or in addition to Sirt1 activation are likely to be responsible for the observed synergism. A hypothetical model summarizing mechanisms by which these agents may interact is shown in **Fig. 7**. According to this model, exposure of leukemic cells to resveratrol triggers multiple interrelated actions, including activation of Sirt1 (Milne et al., 2007), induction of ROS (Chandra, 2009), and synchronization of cells in S-phase (Estrov et al., 2003). Activation of Sirt1 diminishes HDACI-mediated p65 acetylation and NF-κB activation, events known to promote HDACI lethality (Dai et al., 2005). Importantly, resveratrol-mediated ROS generation, in cooperation with HDACIs (Insinga et al., 2005), triggers up-regulation of death receptors (e.g., DR5) (Hussain et al., 2011), leading to activation of the extrinsic cascade, release of mitochondrial death proteins (e.g., cytochrome c), and full engagement of the apoptotic cascade. In addition, ROS also causes DNA damage, the lethal consequences of which may be exacerbated by HDACI-mediated interference with or down-regulation of DNA repair proteins (Subramanian et al., 2005;Lee et al., 2010). Finally, S-phase synchronized leukemic cells may be particularly susceptible to the oxidative injury (Ge et al., 2006) and DNA damage triggered by the preceding events. The net effect of these cooperative actions is a pronounced induction of cell death. Given the relative lack of toxicity of resveratrol concentrations considerably higher than those employed in the present study (Howells et al., 2011), a strategy combining HDACIs with resveratrol warrants further attention in AML and possibly other hematologic malignancies.

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Conducted experiments: Yaseen, Chen, Hock, and Dai.

Contributed new reagents or analytic tools: Chen, Dai, and Grant.

Performed data analysis: Yaseen, Chen, and Dai.

Wrote or contributed to the writing of the manuscript: Yaseen, Dai, and Grant.

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Footnotes

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Legends for Figures

Figure 1. *Resveratrol interacts synergistically with LBH-589 in human AML cell lines and primary AML blasts*. U937 cells were exposed to (A) 50 μM resveratrol +/- 10 - 20 nM LBH-589 for 24 hr; (B) 10 - 50 μM resveratrol +/- 15 nM LBH-589 for 24 hr; or (C) 50 μM resveratrol +/- 15 nM LBH-589 for 4 – 48 hr. (D) MV-4-11 cells were incubated with 25 μM resveratrol +/- 7.5 nM LBH-589 for 24 hr. After treatment, the percentage of cell death was determined by 7AAD staining and flow cytometry. Values = the means ± S.D. for triplicate determinations performed on three separate occasions (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. each agent alone). (E) U937 were treated with LBH-589 +/- resveratrol at a fixed ratio (1: 2000) for 24 hr, after which apoptosis was monitored by annexin V/PI analysis. (F) Blasts from the bone marrow (BM) of a patient with AML were exposed to 50 μM resveratrol +/- 10 nM LBH-589 or 1 μM vorinostat for 24 hr, after which cell death was analyzed by Annexin V-FITC/PI staining and flow cytometry. Upper and lower right panels correspond to annexin$^+$/PI$^+$ (late cell death) and annexin$^+$/PI$^-$ (early cell death) respectively. An additional experiment yielded equivalent results.

Figure 2. *Resveratrol/HDAC inhibitors induce DNA damage, mitochondrial injury, and caspase activation in human leukemia cells*. (A) U937 cell cells were incubated with 50 μM resveratrol +/- 15 nM LBH-589 for the indicated intervals (left panel); MV-4-11 cells were exposed to 25 μM resveratrol +/- 7.5 nM LBH-589 for 24 hr (right panel). After treatment, cells were lysed and Western blot analysis performed to monitor expression of γH2A.X. (B) U937 cells were treated with 50 μM resveratrol +/- 15 nM LBH-589 or 1.5 μM vorinostat for the indicated intervals, after which S-100 cytosolic fraction was prepared and subjected to Western blot analysis to monitor release of mitochondrial cytochrome c into cytosol. (C) U937 cells were treated for 8 hr as described in 2B, after which comet assays were performed to assess single- and double-stranded DNA breaks. (D-E) U937 (D) and MV-4-11 (E) cells were exposed to resveratrol (U937, 50 μM; MV, 25 μM) +/- LBH-589 (U937, 15 nM; MV, 7.5 nM) or vorinostat (U937, 1.5 μM; MV, 1 μM) for 24 hr, after which cleavage of caspases-8, -3, -9, and PARP was assessed by Western blot analysis. CF = cleaved fragment. Each lane was loaded with 30 μg of protein; blots were stripped and reprobed with β-actin to ensure equivalent loading and transfer.

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Figure 3. Resveratrol inhibits HDACI-induced RelA/p65 acetylation and NF-κB activation. (A) U937 cells were incubated with 50 μM resveratrol +/- 15 nM LBH-589 or 1.5 μM vorinostat for 8 hr, after NF-κB activity was determined by an ELISA-based p65-specific NF-κB activity assay. Values represent the means ± S.D. for triplicate determinations performed on three separate occasions. (B) U937 cells were treated as described in (A) for 24 hr, and Western blot analysis was performed using antibodies specifically recognizing lysine 310 acetylated p65. Total p65 was probed for comparison. * = nonspecific bands. (C) Alternatively, cells were harvested and lysed at the indicated intervals, and 200 μg protein was subjected to immunoprecipitation (IP) with anti-p65 antibody, followed by Western blot analysis using anti-lysine antibody. IgG (H) = IgG heavy chain. (D) U937 cells were stably transfected with constructs encoding shRNA specifically targeting Sirt1 (shSirt1) or a scrambled sequence as negative control (shNC). Western blot analysis was performed to determine down-regulation of Sirt1 protein in two shSirt1 clones (designed 36 and 45), compared to parental U937 and shNC cells. Cells were then exposed to 15 nM LBH-589 or 1.5 μM vorinostat for 48 hr, after which cell death was monitored by 7AAD staining and flow cytometry. Values represent the means ± S.D. for triplicate determinations performed on three separate occasions. n.s. = not significant ($P > 0.05$). For Western blot analysis, each lane was loaded with 30 μg of protein; blots were stripped and reprobed with β-actin to ensure equivalent loading and transfer.

Figure 4. *The resveratrol/HDACI regimen induces ROS production, resulting in increased DR5 expression, caspase-8 activation, and DNA damage*. (A) U937 cells were incubated with 50 μM resveratrol +/- 15 nM LBH-589 (left panel) or 1.5 μM vorinostat (right panel) for the indicated intervals, after intracellular ROS levels were monitored by H_2DCFDA, a cell-permeable indicator for reactive oxygen species, staining and flow cytometry. Values represent the means ± S.D. for triplicate determinations performed on three separate occasions. (B-C) U937 cells were treated with 50 μM resveratrol +/- 15 nM LBH-589 or 1.5 μM vorinostat in the absence or presence of 400 μM MnTBAP for 6 hr, after which ROS was measured as described in 4A (B), and cell death was determined by 7AAD staining and flow cytometry (C). Values represent the means ± S.D. for triplicate determinations performed on three separate occasions. *** $P < 0.001$, compared to the same treatment without MnTBAP. (D-E) U937 cells were treated with 50 μM

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resveratrol +/- 15 nM LBH-589 or 1.5 μM vorinostat after pre-treatment with 5 μM ML171 (D) or 5 mM sodium azide (Az, E) for 24 hr, after which cell death was determined by 7AAD staining and flow cytometry.

Figure 5. *Dominant-negative caspase-8 blocks cell death induced by the resveratrol/HDACI regimen*. (A-B) U937 cells were treated with 50 μM resveratrol +/- 15 nM LBH-589 or 1.5 μM vorinostat in the absence or presence of 400 μM MnTBAP for 24 hr, after which cells were lysed and subjected to Western blot analysis to monitor expression of DR5, γH2A.X, and caspase-8. Each lane was loaded with 30 μg of protein; blots were stripped and reprobed with β-actin to ensure equivalent loading and transfer. CF = cleaved fragment. (C) U937 cells stably transfected with dominant-negative (DN) caspaspe-8 or its empty-vector control (pcDNA3.1) were treated with 50 μM resveratrol +/- 15 nM LBH-589 or 1.5 μM vorinostat for 24 hr, after which Western blot analysis was performed to monitor cleavage/activation of caspase-8 and -3. CF = cleaved fragment. Blots were stripped and re-probed with antibodies to β-actin to ensure equivalent loading and transfer. (D) Alternatively, flow cytometry was performed to assess cell death after 7AAD staining. Values represent the means ± S.D. for triplicate determinations performed on three separate occasions.

Figure 6. *Resveratrol induces S phase accumulation, an effect abrogated by HDACIs*. (A) U937 cells were exposed to 50 μM resveratrol +/- 1.5 μM vorinostat for the indicated intervals, after which flow cytometry was performed to analyze cell cycle distribution after PI staining (upper panels). (B-C) Alternatively, flow cytometry was performed to determine the S phase-specific population by Click-iT™ EdU CellCycle 488-red (7-AAD) Assay Kit (B), or apoptosis in the S phase population (C). Values indicate percentage of S phase (EdU^+) cells (B) or percentage of apoptotic (Annexin V-APC^+) cells in the S phase (EdU-$AF488^+$) population (C).

Figure 7. *A theoretical model of synergistic interactions between resveratrol and HDAC inhibitors*. Resveratrol triggers multiple interrelated actions in human leukemia cells, including Sirt1 activation, ROS induction, and S phase synchronization. Activation of Sirt1 blocks cytoprotective NF-κB activation by preventing HDACI-mediated p65 acetylation. In addition,,

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resveratrol induces ROS generation, which in conjunction with HDACIs, promotes up-regulation of DR5 and activation of the extrinsic apoptotic cascade, followed by release of mitochondrial death proteins (e.g., cytochrome c), thus fully engaging apoptotic signaling cascades. Alternatively, ROS may directly cause mitochondrial damage. Finally, resveratrol synchronizes cells in S phase, which may be particularly susceptible to HDACI- and/or ROS-mediated DNA damage, which contributes to activation of cell death pathways. Solid line = pro-death signal; dashed line = pro-survival signal.

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Figure 1

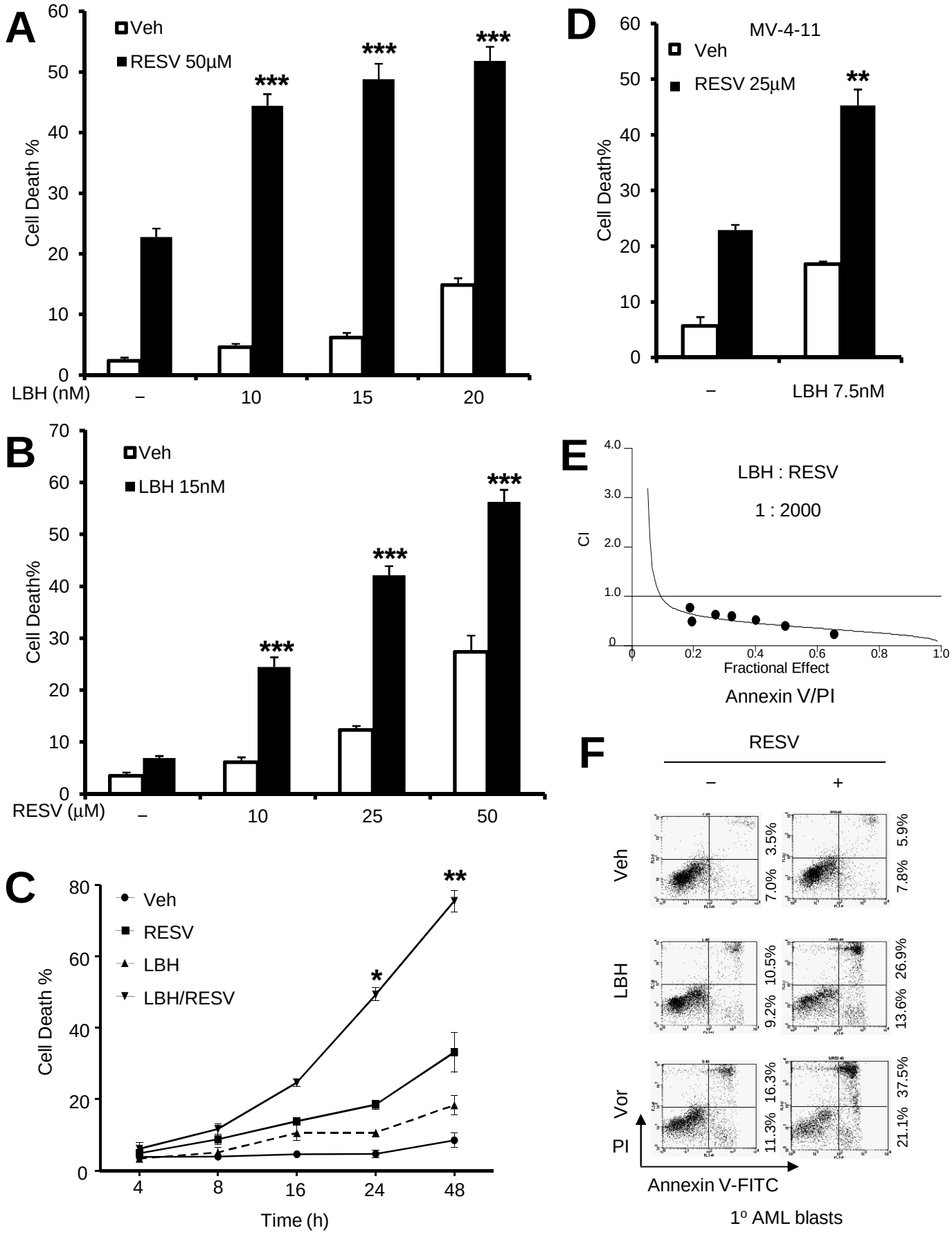

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Figure 2

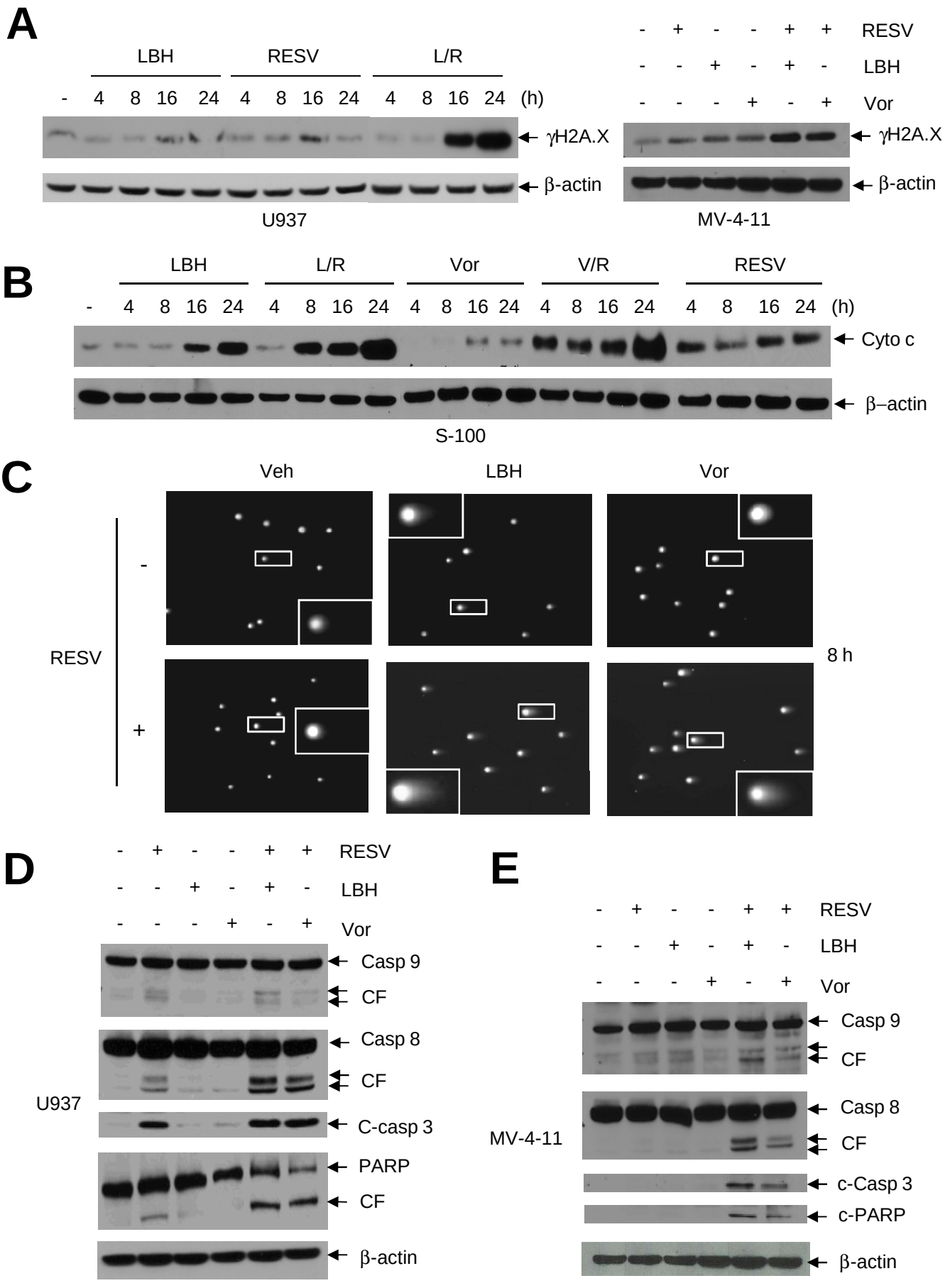

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Figure 3

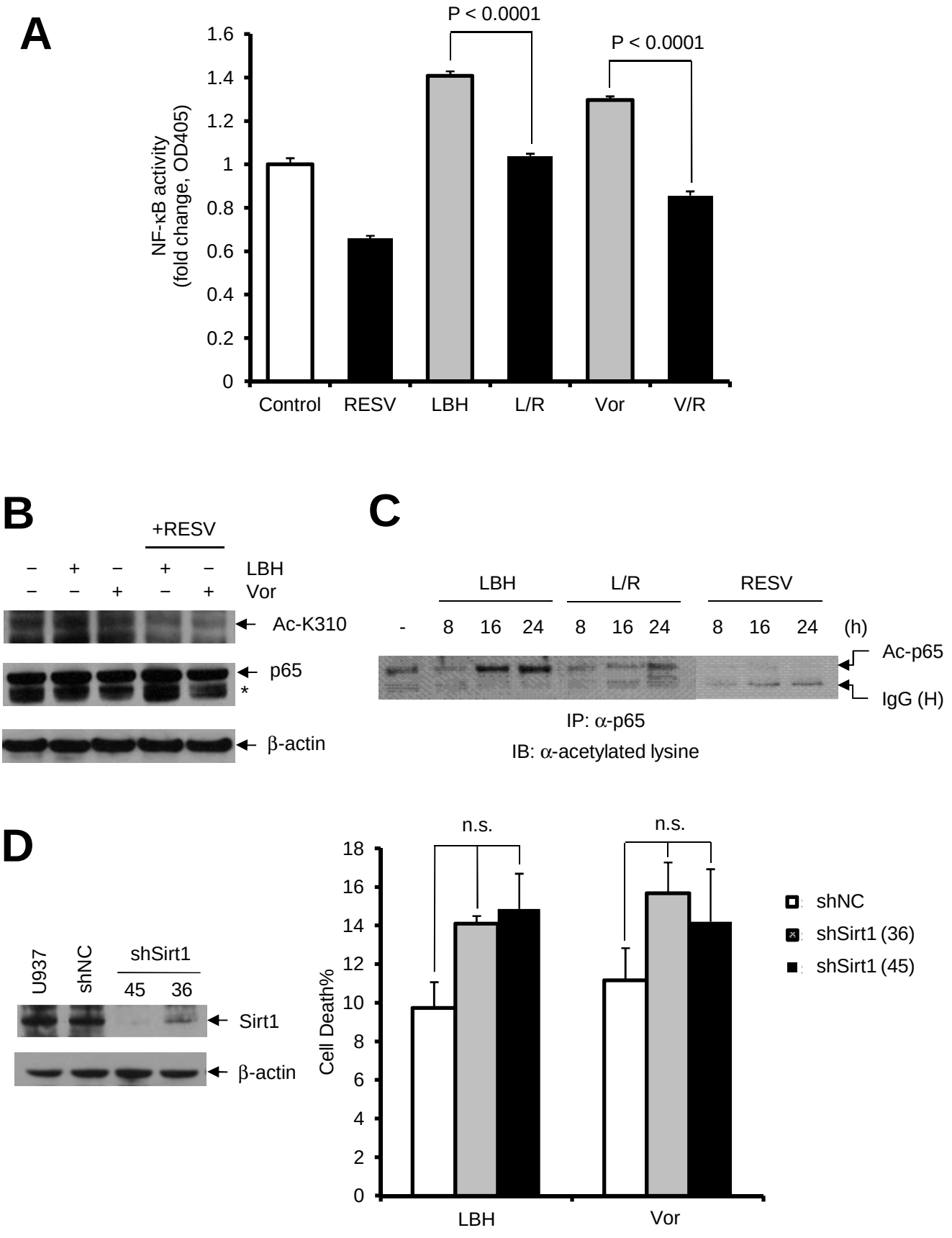

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Figure 4

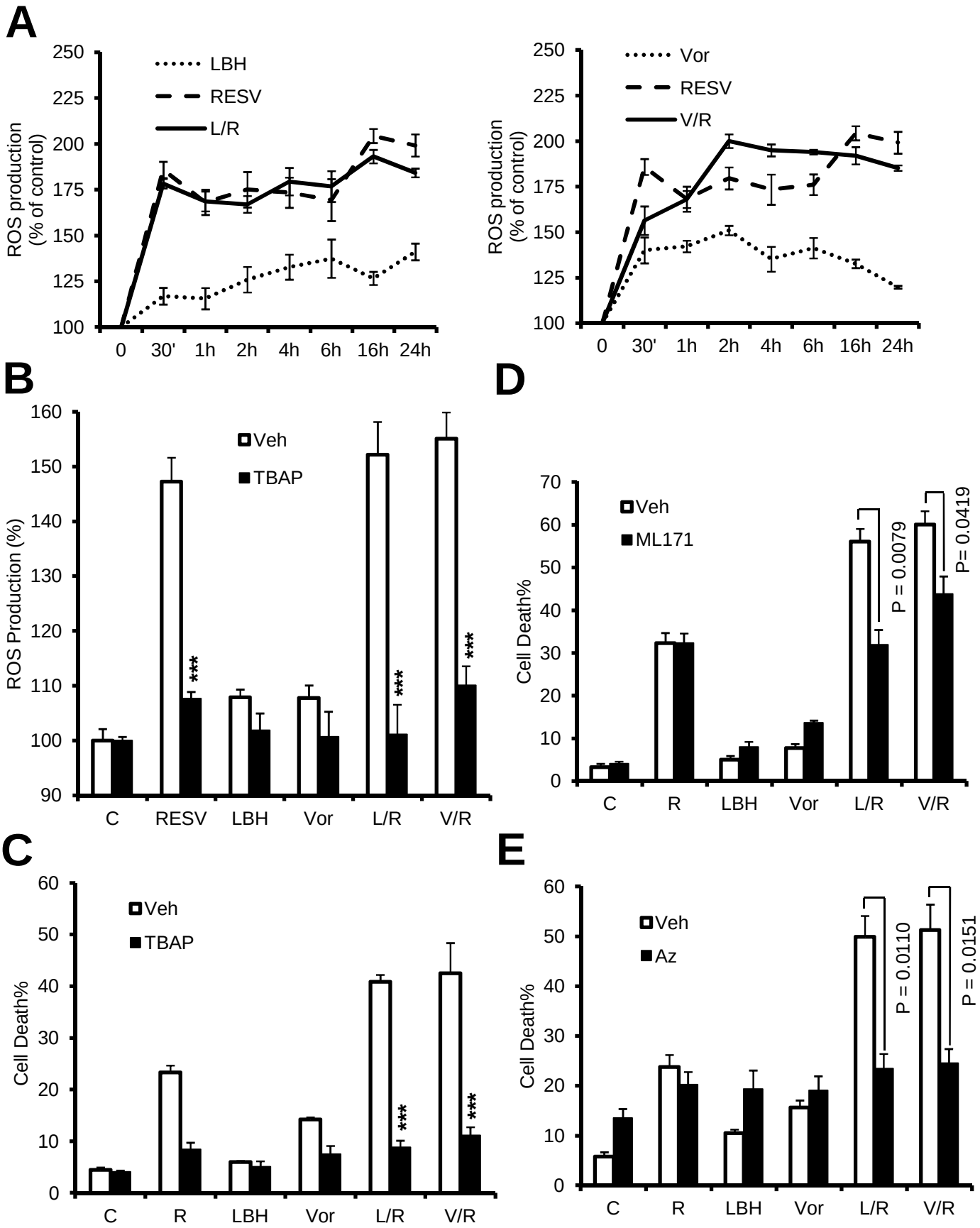

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Figure 5

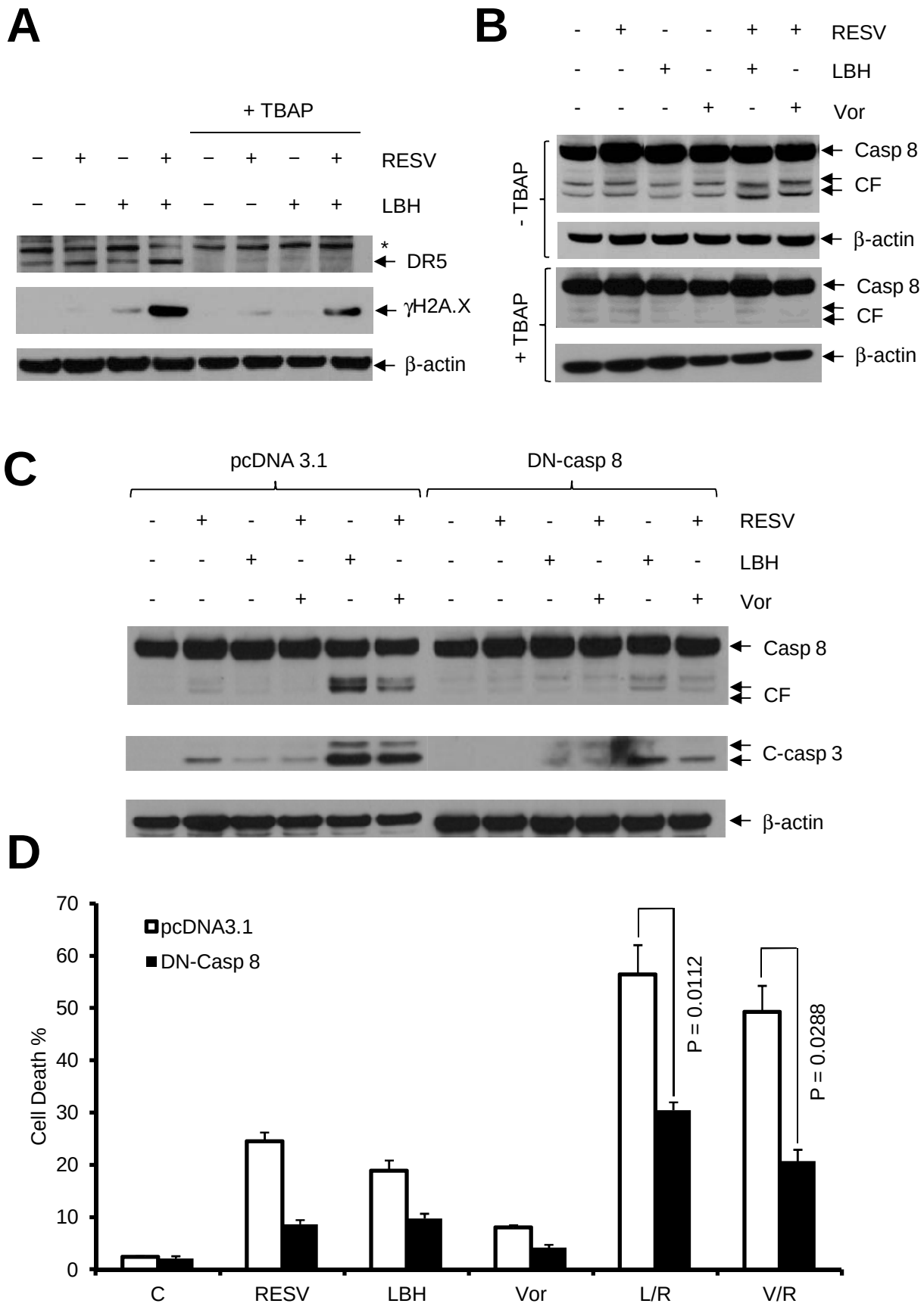

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Figure 6

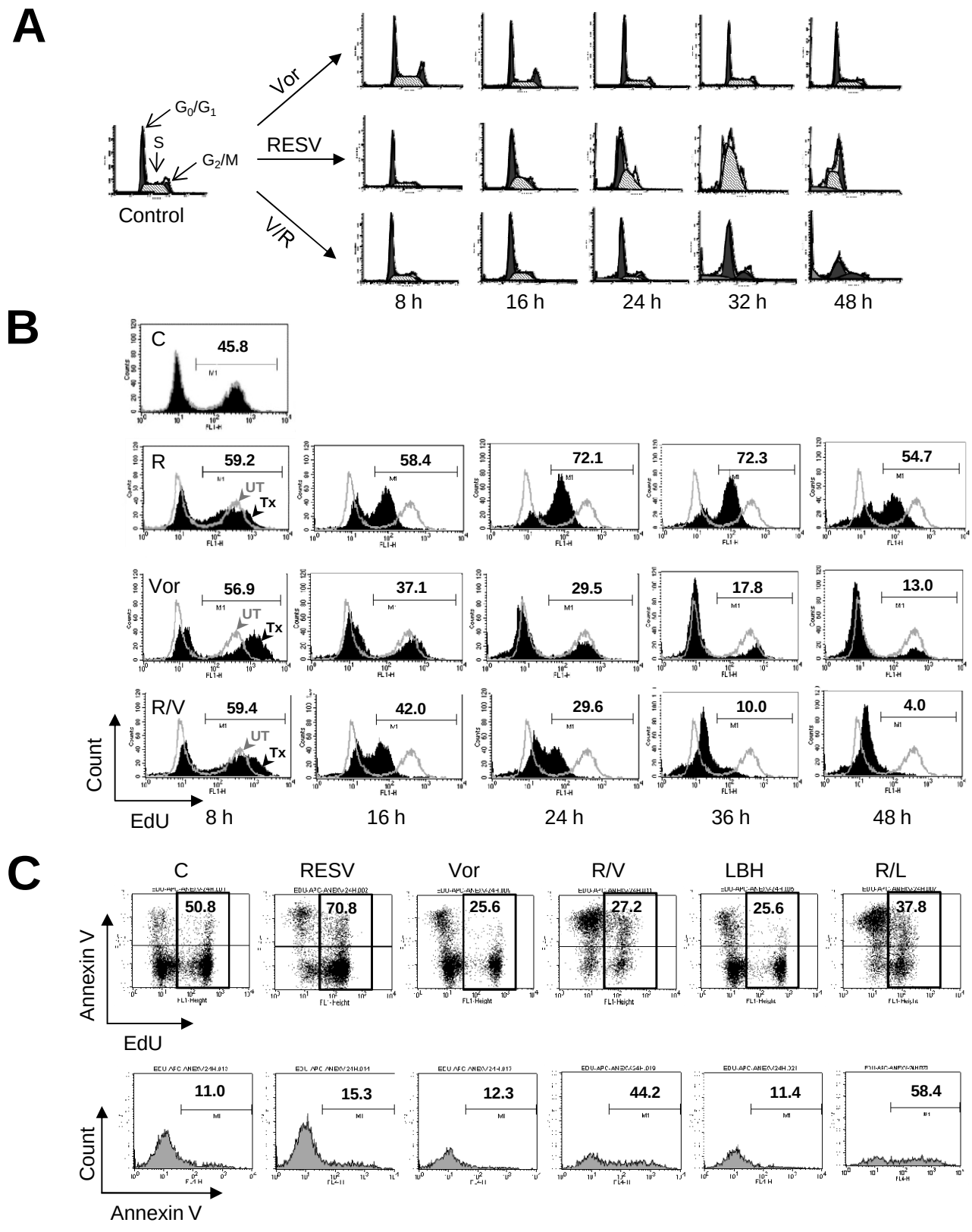

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Figure 7