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THE NATURAL POLYPHENOL RESVERATROL POTENTIATES THE LETHALITY OF HDAC INHIBITORS IN ACUTE MYELOGENOUS LEUKEMIA CELLS THROUGH MULTIPLE MECHANISMS.

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Introduction

1. Leukemia.

Leukemia is a broad term covering a spectrum of blood or bone marrow cancers characterized by an abnormal increase of blood cells. Leukemia is classified according to pathological and clinical criteria into: Acute with rapidly progressing course and predominance of blasts cells, or chronic signifying slower disease progression with greater number of mature cells. Another classification is according to the affected blood cell type: lymphocytic leukemia and myeloid (myelogenous) leukemia. Combination of these two classifications generates four main categories with other subcategories included within each category or outside these classifications. Acute lymphoblastic leukemia (ALL)(1) is malignant disease involve lymphocyte cell precursors. ALL is the most common type affecting children and adolescents; it is the main cause of cancer related mortality in this group. The diagnosis of ALL depends on immunophenotyping “as the leukemic lymphoblasts lack specific morphologic or cytochemical features”.(2) Chronic lymphocytic leukemia (CLL): this malignant disease involves B lymphocyte lineage; it is more common in adult over 40 years old, the median age at diagnosis is 72 years(3). The majority of the patients are asymptomatic at presentation(4). Men affected more than women. CLL is the most common form of adult leukemia in Western countries (5). Chronic myelogenous leukemia (CML) is a myeloproliferative disorder occurs mainly in adults. It is characterized by a cytogenetic aberration consisting of a reciprocal translocation between the long arms of chromosomes 22 and 9; t(9;22) Philadelphia (Ph) chromosome results in formation of BCR/ABL fusion gene encodes a chimeric protein with tyrosine kinase activity, treatment is with imatinib (Gleevec) targets this mutation(6). One subtype of CML is chronic monocytic leukemia. Hairy cell leukemia (HCL) is sometimes considered a subset of CLL. T-cell prolymphocytic leukemia (T-PLL) mature T cell leukemia is a very rare and aggressive leukemia affecting adults. Large granular lymphocytic leukemia may involve either T-cells or NK cells; it is a rare and indolent (not aggressive) leukemia. Adult T-cell leukemia is caused by human T-lymphotropic virus (HTLV), a virus similar to HIV. HTLV infects CD4+ T-cells and replicates within them, it does not destroy CD4+ cells like HIV, and HTLV "immortalizes" the infected T-cells.

Acute myelogenous leukemia (AML) is a malignant disease of the hematopoietic precursors in the bone marrow. It is characterized by the presence of more than 20% blast cells in the bone marrow. Subtypes of AML include acute promyelocytic leukemia, acute myeloblastic leukemia, and acute megakaryoblastic leukemia (7). AML is an aggressive leukemia with variable phenotypic presentation and genetic background. AML affects primitive hematopoietic stem cells and transforms them into leukemic stem cells. A two-hit model is responsible for AML development in which first hit mutations (such as FLT3-ITD) affect proliferation or result in survival, while the second mutations block myeloid cell differentiation and allow self-renewability (8). FLT3 is a membrane-bound receptor tyrosine kinase (RTK) the FMS-like tyrosine kinase 3 (*FLT3*) gene is located on chromosome 13q12, the duplication of the FLT3 gene and point mutations of the N-RAS gene are the most frequent somatic mutations in acute myeloid leukemia (AML) (8) FLT3 mutation found in 25–45% of all AML patients (9)

2. HDAC (histone deacetylases)

2.1. Class I, class IIa, class IIb, class III (SIRT-1) Histone deacetylase (HDAC) enzymes catalyze the removal of the acetyl group from Lysine Amino acid residues on the histone tail leading to suppression of transcription by condensing the chromatin structure(10). HDAC are subdivided into four classes based on their homology to yeast HDACs, their subcellular localization and their enzymatic activities (11). Class I HDACs (HDACs 1, 2, 3, and 8) are similar to the yeast RPD3 protein and localize to the nucleus. And they show ubiquitous expression in various cell lines and tissues. Class II HDACs (HDACs 4, 5, 6, 7, 9, and 10) are homologous to the yeast HDA1 protein and are found in both the nucleus and cytoplasm (11). Class IIb-HDACs, HDAC6 and 10, are found in the cytoplasm and contain two deacetylase domains (10). Class III HDACs (sirtuins) form a structurally distinct class of NAD-dependent enzymes that are homologues of the yeast *Saccharomyces cerevisiae* silent information regulator 2 (Sir2) proteins, According to their homology to yeast HDAC Sir2p. Seven members have been described in humans so far. Class III-HDACs (SIRT1, 2, 3, 4, 5, 6 and 7) require nicotinamide adenine dinucleotide NAD⁺ for their activity to regulate gene expression in response to changes in the cellular redox status. SIRT1 has been shown to interact with and deacetylate p53, resulting in repression of p53- mediated transcriptional activity. As sirtuins are

involved in many physiological and pathological processes, their activity has been associated with the pathogenesis of cancer, HIV, metabolic, or neurological diseases (12). HDAC11 is the only member in Class IV HDAC and contains conserved residues in the catalytic core regions shared by both class I and II mammalian HDAC enzymes. HDAC11 may have distinct physiological roles from those of the other HDACs (13) see Table-1.

Table 1.

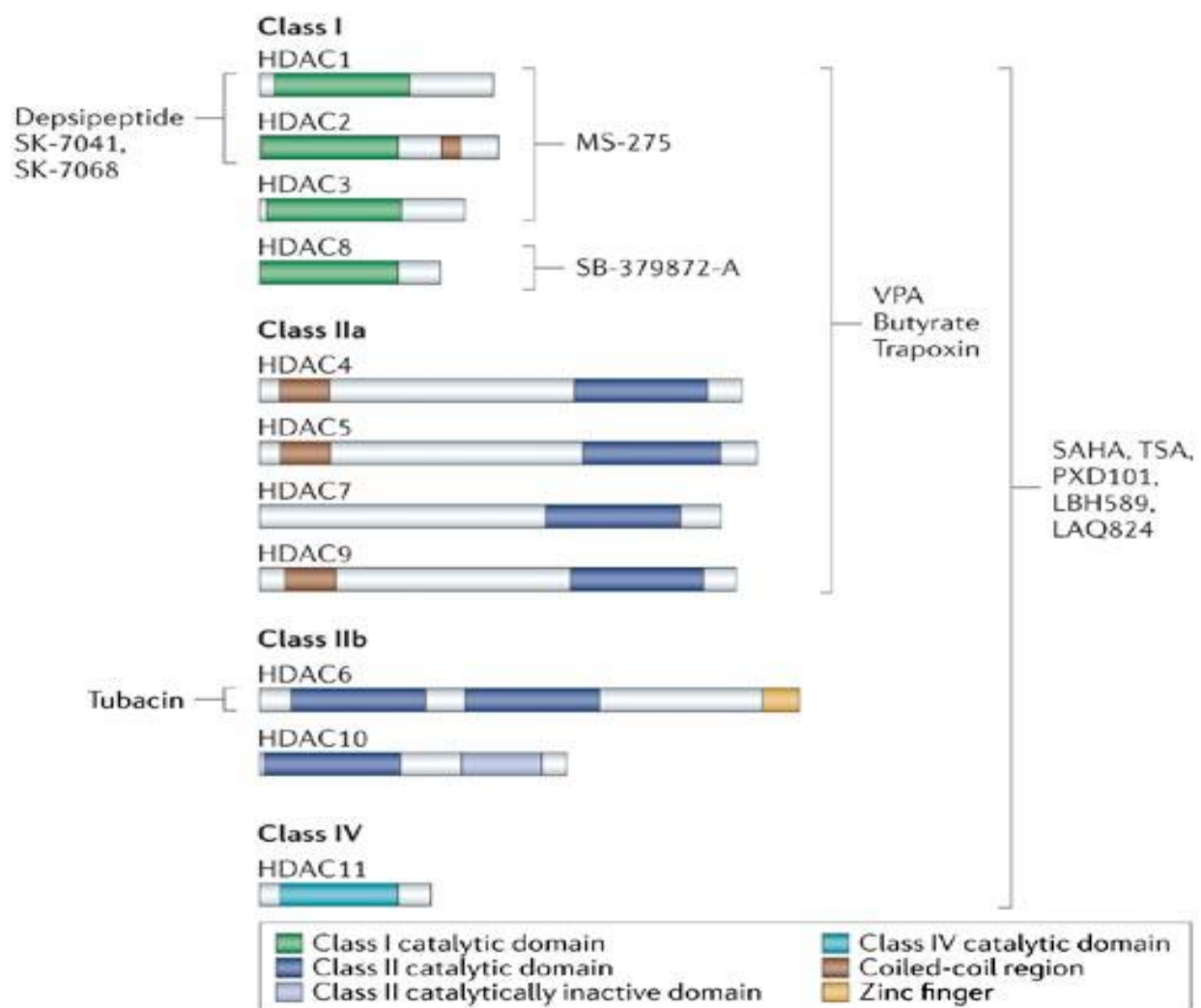
HDACs

Class	Enzymes	Zn ²⁺ Dependent	Localization	Expression
I	HDAC1, HDAC2, HDAC3, HDAC8	Yes	Nucleus	Ubiquitous
IIa	HDAC4, HDAC5, HDAC7, HDAC9	Yes	Nucleus and cytoplasm	Tissue specific
IIb	HDAC6, HDAC10	Yes	Cytoplasm	Tissue specific
III	Sirtuins 1-7	No	Variable	Variable
IV	HDAC11	Yes	Nucleus and cytoplasm	Ubiquitous

- Abbreviation: HDAC, histone deacetylase; Zn, zinc. (14)

2.2. HDAC inhibitors classes (HDACI) Epigenetic modulation of gene expression plays an important role in alterations that along with genetic abnormalities lead to the development of cancer. The accessibility of DNA within chromatin units is governed by multiple histone modifications including methylation(15), phosphorylation(16), ribosylation (17), sumoylation (18), ubiquitination (19), and acetylation (20). The acetylation of histones regulates compaction of DNA. This compaction blocks gene transcription and inhibits differentiation, providing a rationale for developing HDAC inhibitors. HDAC inhibitors promote growth arrest, differentiation, and apoptosis of tumor cells, with minimal effects on normal tissue (14). Multiple HDACI classes have been identified which variably inhibit class I and II HDAC catalytic domains (21). They include short-chain fatty acids (butyrate, valproic acid), hydroxamic acids (SAHA, CBHA, LAQ824, TSA) which are pan-inhibitors for class I, II and IV. Other HDACIs are more specific like benzamides (MS-275, CI-994), and cyclic peptides (depsipeptide) (21, 22). HDACIs such as butyrate also long been Known to induce leukemic cell maturation (23), and second-generation HDACIs such as SAHA, MS-275, and LAQ824, which are 3-4 logs more

potent than butyrate, exhibit similar capacities (24-26) . HDACI-mediated leukemic cell maturation is associated with growth arrest resulting in CDKI p21^{CIP1} (27) promoter acetylation (27) that's leads to p21^{CIP1} induction. Moreover, HDACIs induce apoptosis at higher concentrations (24). The common mechanism of action of HDACI is to bind a critical Zn²⁺ ion required for catalytic function of the HDAC enzyme. However, HDACI lethality may correlate poorly with histone acetylation status (25). Such findings suggest that HDACI-mediated acetylation of non-histone proteins, may play a critical role in the antileukemic activity of these compounds. Several different chemical compounds have been shown to inhibit class III sirtuins deacetylase activity such as Splitomicin which inhibits the NAD⁺-dependent deacetylase activity of Sir2 in vitro and sirtinol which is derived from 2-hydroxy-1-napthaldehyde. Sirtinol interferes with body axis formation in *Arabidopsis* (28), other sirtuins Inhibitors include a product of the Sir2 deacetylation reaction (Nicotinamide) Nicotinamide has been shown to inhibit a Sir2 homolog, SIRT1, a negative p53 regulator, promoting p53-dependent apoptosis in mammalian cells (28).



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Fig.1

2.2.1. HDACIs mechanisms of lethality:

Multiple mechanisms of HDACI lethality have been identified (21, 29):

1. Perturbations in cell cycle regulation (30); For example, HDACIs can disrupt cell cycle checkpoints, resulting in premature exit from mitosis and cell death (31). Furthermore, dysregulation of HDACI-mediated p21^{CIP1} induction dramatically lowers the cell death threshold(32, 33)

2. modulation of stress and survival signaling pathways(34) ; and interference with chaperone function(35, 36); Attention has also focused on the acetylation of Hsp90 by HDACIs and disruption of its chaperone function, leading to degradation of multiple pro-survival client proteins, including Raf, ERK, Erb2, Bcr/Abl, among others (26, 35). In particular, HDACI-mediated inhibition of the class IIb HDAC6 has been implicated in a) Hsp90 acetylation(37) and b) acetylation of cytoskeletal proteins such as tubulin(38) as well as the dynein motor, which disrupts aggresome formation and leads to apoptosis(39) Significantly, mutant oncogenic proteins appear to be particularly susceptible to degradation associated with Hsp90 dysfunction(40).
3. Activation of death receptor pathways (41); HDACIs activate the extrinsic apoptotic pathway (42) by up-regulating death receptors and ligands, including DR4, DR5, Fas, and FasL(41).
4. Induction of mitochondrial injury (43); epigenetic changes induced by HDAC Is, lead to the expression of a protein that facilitates the pathway by which mitochondria activate caspase-3 and trigger apoptotic death of lymphoid and colorectal cancer cells (43).
5. Antiangiogenic actions; HDAC I suppresses angiogenesis likely by preventing endothelial cells from responding to the angiogenic stimulus by altering VEGF signaling (44).
6. Generation of ceramide (45). HDACI lethality, either alone (45) or with cytotoxic drugs (46) has been linked to generation of the pro-apoptotic lipid second messenger ceramide.
7. Induction of oxidative damage (42); In eukaryotic cells, partially reduced and reactive O₂ metabolites form during oxidative metabolism and in response to diverse environmental stresses(47). Such metabolites, collectively referred to as reactive oxygen species (ROS), include superoxide anion (O₂•⁻), hydrogen peroxide (H₂O₂), and hydroxyl radicals (•OH)(48), and injure cells via DNA damage, lipid peroxidation, and other actions(49). In addition to their capacity to damage cells directly, there is mounting evidence that ROS play important roles in cell signaling (50). Cellular defensive mechanisms against ROS include induction of anti-oxidant enzymes(51), this induction is dependent upon NF-κB-mediated transcription(52, 53). Conversely, NF-κB pathway disruption promotes oxidative damage-induced apoptosis (52, 54). Accumulating evidence indicates that perturbations in cellular redox status stemming from alterations in NF-κB activation may represent key determinants of HDACI actions (55).

2.2.1.1. NF-κB and cellular redox status NF-κB represents a family of dimeric transcription factors (RelA/p65, RelB, c-Rel, p50, and p52) implicated in cell proliferation, differentiation, inflammation, survival, and response to environmental stresses, particularly oxidative

damage(56). Three major NF- κ B pathways have been identified: a) the canonical pathway, triggered by pro-inflammatory cytokines (e.g., TNF α), and primarily associated with RelA/p50 heterodimers(57). b) The non-canonical pathway, activated by BAFF or CD40 ligand (58). c) The atypical pathway, associated with DNA damage (59). NF- κ B is regulated through cytoplasmic sequestration by members of the I κ B family, including I κ B α , β , ϵ , λ ,BNS, and Bcl-3, p100, and p105(57). In the canonical pathway, diverse stresses (e.g., TNF α) activate IKKs and phosphorylate I κ B family members, particularly I κ B α , which bind and trap NF- κ B in the cytoplasm(60). Upon phosphorylation I κ B α is ubiquitinated and proteasomally degraded, allowing NF- κ B transport to the nucleus where it binds to DNA consensus sequences and promotes transcription of survival-related genes, including Bcl-x_L, XIAP, FLIP, and A1, among others(61). The most abundant and intensively studied NF- κ B family member is RelA/p65(62). Significantly, NF- κ B downstream targets include genes encoding enzymes critically involved in redox homeostasis (52, 53). alterations in NF- κ B status have been implicated in inflammation-associated transformation(63), chemoresistance of tumor cells (64), and HDACI actions(65). Notably, the NF- κ B pathway plays a key role in oxidative stress defenses (66), and represents a critical mediator of leukemic cell survival (67), including that of leukemic stem cells (LSC) (68).

2.2.1.2 Role of NF- κ B acetylation and ROS generation in determining the lethality of

HDACI The resistance of cells to HDACI exposure depends on the extent of induction of ROS damage (25, 42). It also depends on survival signaling pathways status, such as NF- κ B (34, 69) For example, cell death induction by the second generation HDACIs SAHA and MS-275 has been directly linked to the generation of ROS (42). HDACIs can induce initial NF- κ B activation via early IKK, and I κ B alpha degradation (70). Moreover, sustained NF- κ B activation involves other events e.g., RelA acetylation (71). Mayo et al., have shown that HDACIs trigger NF- κ B activation through an Akt-dependent and p300-dependent process involving acetylation of RelA/p65 on lysine 221 and 310 residues (72). Liu et al., reported that SAHA triggers the AKT-dependent-phosphorylation of p300, resulting in enhanced RelA/p65 acetylation and transcriptional activity (73). this findings consistent with those of Greene et al., who reported that RelA/p65 acetylation, a nuclear event mediated by class I HDACs (e.g., HDAC3)(74) diminished I κ B alpha binding and prevented nuclear export of NF- κ B; which also increased NF- κ B DNA binding/activation(75). Importantly, these events are associated with attenuation of HDACI lethality (72). Thus, the pro-apoptotic actions of HDACIs as discussed above may be

intrinsically self-limited by RelA/p65 acetylation or other mechanisms responsible for sustained activation of the NF- κ B axis.

2.2.2. Clinical significance of HDACI: More than 15 HDAC inhibitors have been tested in preclinical and early clinical studies. (14). Following FDA approval of vorinostat (suberoylanilide hydroxamic acid, or SAHA, a hydroxamic acid derivative that inhibits both class I and II HDACs) in 2006 (76), several novel HDAC inhibitors (HDACIs) have entered clinical trials, (77, 78) HDACI have also been evaluated in clinical trials in the last few years. The affects of these agents whether alone or in combination with other drugs observed in many hematologic malignancies including T-cell lymphoma, Hodgkin lymphoma, and myeloid malignancies (79). Panobinostat (LBH589) is another novel HDACI that showed the proliferation inhibition and apoptosis induction in fifteen patients with different refractory hematologic malignancies (80). But an asymptomatic cardiac arrhythmia result in early discontinuation of the study. In another phase II study intermittent dose scheduling shown to minimize this cardiac side affect (81). HDACIs acute toxicities ranging from general fatigue, gastrointestinal symptoms, to transient cytopenias, with no significant observed long-term toxicities. (77). The major clinical challenges that limit the successful HDACI development and use include: the spectrum of these agents activity, choosing the best strategy in solid tumors, agent toxicity, particularly the cardiac side effects, and the biological markers that should be used in HDACIs clinical applications (78)

2.3. HDAC Class III agonist (Resveratrol)

Many activators that stimulate the SIRT1 deacetylase activity were recently identified. Of these, two small structurally similar molecules of these activators, quercetin and piceatannol, can stimulate SIRT1 activity more than several fold (28). One of the most potent activator of Class III is Resveratrol (28). Resveratrol (3,5,4_-trihydroxystilbene), is a phytoalexin compound found in grapes, red wines, berries, and peanuts (82), implicated in a number of health benefits., including cancer (83-85). Resveratrol lowers the *K_m* values for both the acetylated peptide and NAD (28). The number and position of hydroxyl groups as well as intramolecular hydrogen bonding are essential features in determining the biological activity of Resveratrol and other Polyphenol (86). See figure-2

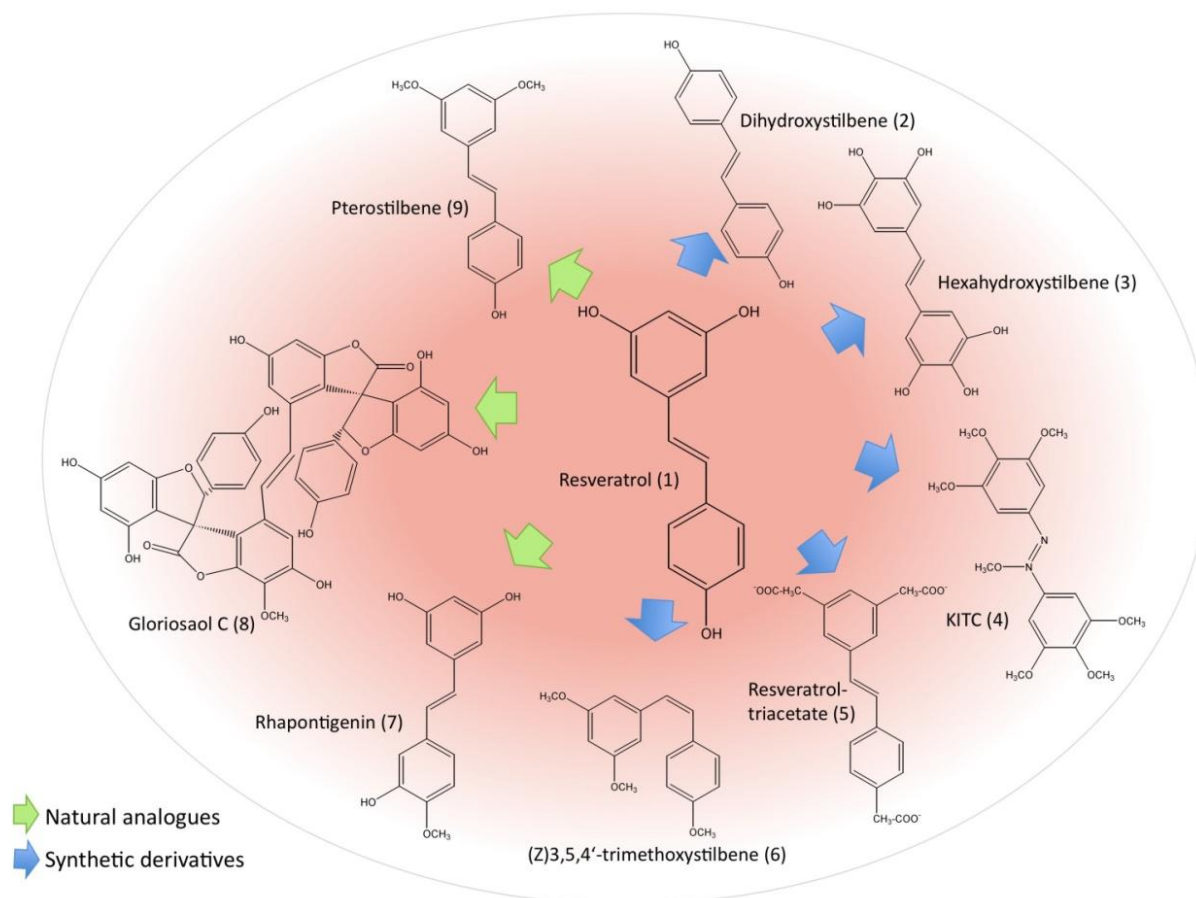


Fig.2 (86)

2.3.1. Therapeutic use of Resveratrol

Resveratrol was invoked to explain the reversed correlation between coronary heart disease and wine consumption in what's known as the "French Paradox." (87). Wine and grape extracts have been long shown to decrease platelet aggregation (88). Interestingly, Resveratrol prevents platelet aggregation in vitro (89), and in vivo (90). Also, Resveratrol is capable of relaxing isolated arteries through multiple pathways (91, 92) and can also suppress pathological increases

in the peroxidation of lipids to exert antioxidant effects that decrease the risk of coronary artery disease and LDH oxidation (93). Moreover, Resveratrol has role in cholesterol and triglycerides deposition (94). As discussed previously, Resveratrol exhibit activating role on Sirtuins and HDAC III which play a significant role in promoting survival in response to environmental conditions(85). In yeast, worms and flies, sirtuins are associated with extended lifespan(85) Resveratrol extends the life spans of *S. cerevisiae* (95) *Caenorhabditis elegans* and *Drosophila melanogaster* (96) and short-lived fish (97). However, the role of Resveratrol in increasing mammalian lifespan is still questionable (85). Other *in vivo* effects of Resveratrol include, protection against hearing loss (98), diabetes (improving glucose, triglyceride metabolism and insulin sensitivity in diabetic rates), pathological inflammation, and viral infection (85). Resveratrol also reduce injuries to the kidneys (99), spinal cord (100), And liver(85). Lower doses of Resveratrol can penetrating the blood–brain barrier and have neuroprotective effects (85, 101).

2.3.2. Role of Resveratrol in cancer treatment:

In-vitro and *in-vivo* evidence for Resveratrol anti-cancer activity was first reported 14 years ago (102, 103). Since this date, many researchers provided abundant evidence for the use of Resveratrol as a chemopreventive agent and the potential for clinical testing of Resveratrol. The anticancer activities of Resveratrol are mediated through several cell-signaling mechanisms that regulate cell cycle, proliferation, inflammatory response, apoptosis, invasion, metastasis, and angiogenesis of cancer cells. Though In some tumor types, Resveratrol has been shown to be a chemoprotector (104). Ramos *et al.* mentioned out 7 main anticancer effects that Resveratrol and related Polyphenol compound can exert on tumor cells(105) see figure-3:

1. Antioxidant effect
2. Antiproliferative and antisurvival effects
3. Cell cycle arrest
4. Apoptosis
5. Anti-inflammatory effect
6. Antiangiogenic and antimetastatic effects
7. Effects on phase-I and -II enzymes

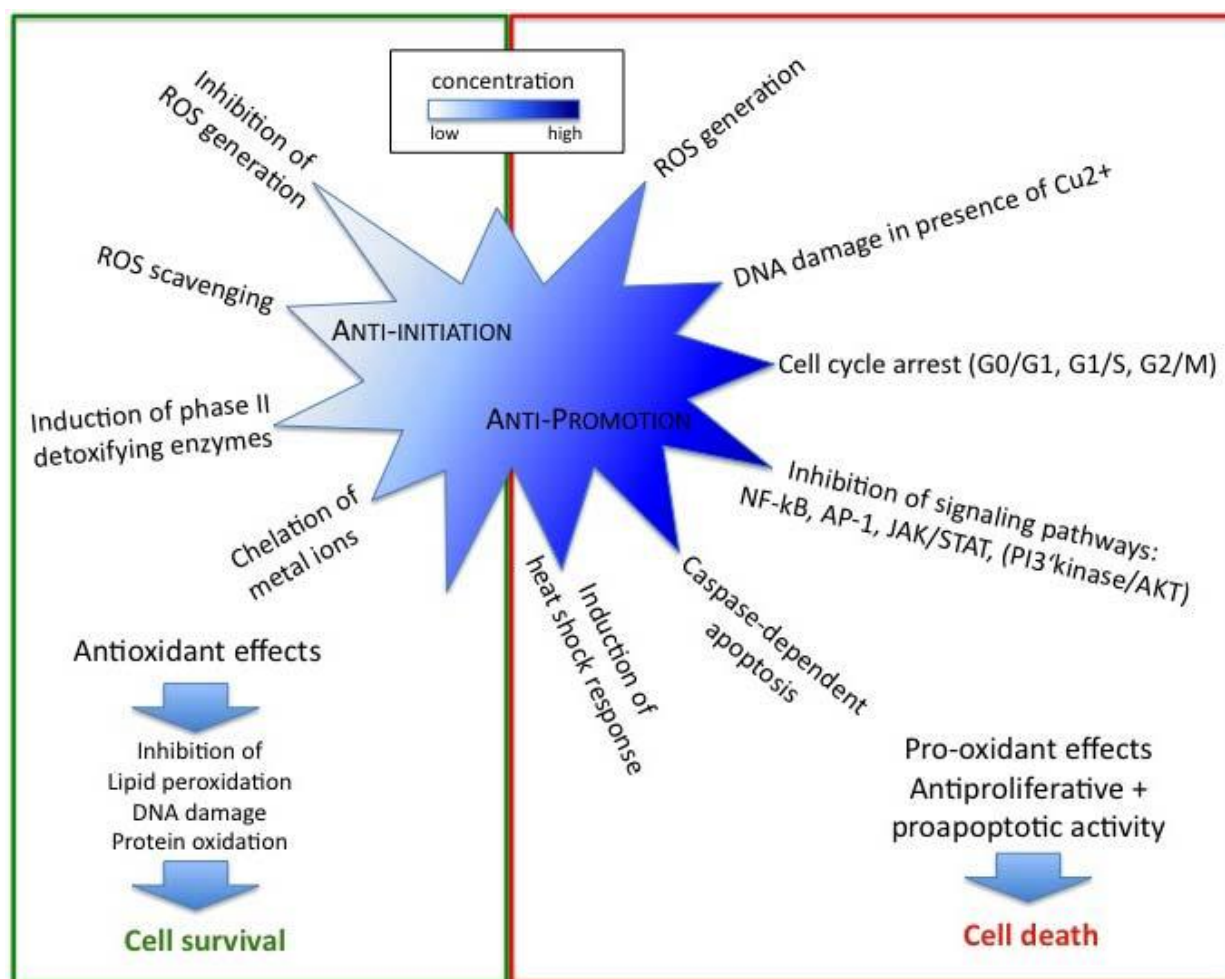


Fig. 3 (86)

➤ Antioxidant effect

Resveratrol can act as antioxidants, preventing injury caused by free radicals and block cancer initiation (105-107). Polyphenol compounds can prevent free radicals injury through different mechanisms: direct radical scavenging(108), chelating divalent cations involved in Fe(II)-dependent apoptosis(109), and modulation of enzymes related to oxidative stress like (glutathione peroxidase (GPx), glutathione reductase (GR), SOD, lipooxygenase, nitricoxide synthase, etc.(105, 110). However, every antioxidant could act as a redox agent who then might act as a pro-oxidant depending on special conditions (111), Dietary Polyphenol can increase ROS production depending on cell type dose and time of treatment (112). Specifically, Resveratrol enhance ROS production in different types of leukemia (111). The pro-oxidant action of Resveratrol “rather than their antioxidant activity” may play a critical role in their anticancer activates, where induction of ROS mediate DNA damages induced apoptosis (113).

Resveratrol binds to DNA in the presence of Cu^{2+} ions and consequently induces DNA damage. In complex with Cu^{2+} , Resveratrol reduces Cu^{2+} to Cu^{+} while the emerging oxidized Resveratrol products further enhance the genotoxicity (86, 114).

➤ **Antiproliferative and antisurvival effects:**

Resveratrol can alter cell death signaling through different pathways: down-regulation of p53 which enhances tumor cell survival, and Bax also enhance cell survival and up-regulate c-FLIP and Survivin which inhibit caspase-8(115) and caspase-9 activity(116) respectively(117). Resveratrol is involved in regulating other signaling pathways that affect apoptotic cell death indirectly such as PI3K/protein kinase B (AKT), GFR/Ras/MAPKs, and to our interest, nuclear factor Kappa B (NF- κ B) (105). Resveratrol regulation of inflammation via Inhibition of Cox-2, may also inhibit tumor cell proliferation, angiogenesis, metastasis(118), and induce apoptosis (119).

➤ **Nuclear factor Kappa B (NF- κ B)**

As discussed above several studies have shown that activation of NF- κ B inhibits HDACI-induced apoptosis. RelA/p65 (NF- κ B) acetylation/activation protects leukemic cells from HDACI-induced lethality. Resveratrol is known to act as an activator of the SIRT1 HDAC (120), which has recently been shown to mediate deacetylation of NF- κ B (121, 122).

➤ **Cell cycle arrest**

The normal regulation of cell cycle machinery is impaired in cancer cells (123). This regulation involves different cell cycle regulatory proteins, including cyclins (cyclin A, B, Ds, or E); cyclin-dependent kinases (CDKs) (CDK 1, 2, 4, or 6); and CDK inhibitors, such as p21WAF1 and p53. Resveratrol and other Polyphenol can cause alteration in many cell cycle-specific proteins that might affect cancer cell growth and proliferation. G1/S and G2/M checkpoints, are also important targets for Polyphenols (119). Long-term administration of sub-apoptotic concentrations of Resveratrol on colonic carcinoma cells resulted in growth arrest caused by a chronically enhanced ROS level and activated the DNA damage checkpoint (86). Specifically, many studies have examined Resveratrol cell cycle effects on different leukemic cell lines through S-phase arrest and inhibition of DNA synthesis (67, 124, 125). Polyphenol treatments have various regulatory actions on cyclins, cyclins-dependent kinase (CDK), pRb, p21, p27 and p53 in leukemic cell lines (126). These findings linked the pro-oxidant activity of Resveratrol and the induction of cell cycle arrest in cancer cells.

➤ **Apoptosis**

Many Polyphenols including Resveratrol exert their anticancer effect through the induction of programmed cell death apoptosis. Moreover, leukemic cells and other cancer cells seem to be more sensitive to these influences than normal cells (127, 128). Resveratrol has been reported to drive apoptosis by activating the intrinsic apoptotic pathway in CD95-signaling-resistant ALL cell lines with minimal affect on normal peripheral blood mononuclear cells, also, leukemic lymphoblasts isolated from pediatric patients with ALL undergo apoptosis when treated with Resveratrol (86, 129, 130) The intrinsic pathway was implicated in HL-60 leukemia cells with caspase-8, BID cleavage, subsequent caspase-3 activation and cytochrome C release (86).

Resveratrol down regulates several antiapoptotic proteins like Bcl-2, Bcl-xL, XIAP and survivin, sensitizing cells to caspase-dependent apoptosis (131). Also Resveratrol has been shown to sensitize tumor cells to the apoptotic potential of other various chemotherapeutic agents in many cancers cell lines including multiple myeloma and T cell leukemia cells (132, 133).

➤ **Anti-inflammatory effect**

Polyphenol has the ability to regulate key inflammatory molecules like TNF (134), cyclooxygenase-2 (COX-2) (135). and NF- κ B (134) as discussed previously. This anti-inflammatory effect may have an impact on growth and development of cancer by inhibiting proliferation, angiogenesis and metastasis.

➤ **Antiangiogenic effects**

Angiogenesis is an important factor in tumor growth and metastasis. Resveratrol and other Polyphenols could effects solid tumor invasion and growth through regulation of angiogenesis. (136).

➤ **Effects on phase-I and -II enzymes**

This feature of Resveratrol and other Polyphenol is linked to their anti oxidant potential. Resveratrol has the ability to induce phase II detoxification enzymes like NAD (P) H (quinone oxidoreductase). Potential of the Dietary Antioxidants Resveratrol and Curcumin in Prevention and Treatment of Hematologic Malignancies (86). other Polyphenols could modulate endogenous antioxidant enzymes (induction of phase II and inhibition of phase I cytochrome P450 enzymes) (119)

2.3.3 Adverse side effect of Resveratrol

Resveratrol chemopreventive potential in *in-vivo* and *in-vitro* is without significant or severe side effects (86). However, Resveratrol may exert an anti-proliferative effect on non-malignant B-cell lymphoblastoid cell line but without causing irreversible cell death (137). Resveratrol doesn't induce primary DNA or chromosomal damage in normal cells but high concentration of Resveratrol may result in slight Increase in chromosomal aberrations (86). Finally, Resveratrol alone exhibit a weak *in vivo* antileukemic response, which might partly be explained by its rapid metabolism (138). The ongoing development and optimization of different Resveratrol derivatives could overcome the bioavailability problem of Resveratrol.

In summary, different HDAC inhibitors induce leukemic cell death through a variety of mechanisms. Leukemic cells respond to this death signal with regulatory changes in many proteins, cell receptors, and transcription factors...etc. One of these responses is leukemic cell up-regulation of survival signaling pathways status NF- κ B that provide leukemic cell with enhanced resistance to HDACI exposure. SIRT1 is a known class III HDAC has recently been shown to mediate deacetylation of NF- κ B and hence deactivation of its survival signal. Resveratrol is a natural polyphenolic compound that has the ability to act as an agonist of SIRT1 HDAC and it has been shown to mediate deacetylation of NF- κ B; In light of the preceding results we were interested in determining what effect Resveratrol might have on HDACI antileukemic actions. We hypothesized that co-administration of Resveratrol and HDACI would result in a diminution in HDACI-mediated NF- κ B acetylation, and a pronounced increase in cell lethality. This rationale is consistent with a model in which p65 (NF- κ B) acetylation/activation protects leukemic cells from HDACI-induced lethality, and lends support for a combination strategy designed to block this process to enhance HDACI antileukemic effects.

2.Methods

2.1 Cells and Cell Culture

U937 cells and MV-411 cells were obtained from American Type Culture Collection (Manassas, VA). Cells were maintained in sterile plastic T-flasks (*Greiner Bio-One*, Frickenhausen, Germany) and placed in a 37°C, 5% CO₂, fully humidified incubator. Cells were counted on a Coulter Counter Cell and Particle Counter, and split when their counts were at least 8.0 x 10⁵ cells/mL, cell cultured in RPMI media containing RPMI 1640 medium supplemented with pen-strep. both provided by (*Gemini Bio-Products, Sacramento, CA*) L-glutamine (*Invitrogen, Carlsbad, CA*), sodium pyruvate (*Mediatech, Manassas, VA.*), non-essential amino acids (*HyClone Logan, UT*). U937 cells stably expressing dominant-negative caspase-8 or their empty vector counterparts were obtained as reported previously (46, 139). U937 shSIRT1 clone and their negative control (NC) counterpart were generated by transfecting U937 Cells with a negative-control small non-specific interfering RNA (siRNA) or siRNA directed against SIRT-1 (*SA Biosciences, Fredrick, MD*) using an *Amaya nucleofector*. Stable clones were selected in the presence of (400 µg/ml) Hygromycin B in case of shSIRT1 clones or (800 µg/ml) G418 sulfate in shNC clones and screened by Western blot analysis for reduced SIRT-1 expression levels compared to those of control U937 cells transfected with non specific shRNA. Cells were left in culture for 24 to 48 hrs prior to exposure to different treatment regimens. Cells were maintained as described above in the presence of the corresponding selection antibiotics.

2.2 Collection and processing of primary cells

Peripheral blood samples were obtained with informed consent from patients with acute myelogenous leukemia (AML) undergoing routine diagnostic aspirations with approval from the institutional review board of Virginia Commonwealth University. Informed consent was provided. AML blasts were isolated and cultured as described (46). CD34⁺ Normal mononuclear cells were also obtained with informed consent from cord blood of samples. CD34⁺ cells were isolated using an immunomagnetic bead separation technique as described (140). Cells were then re-suspended in fresh medium, and prepared for studies as described above.

2.3 Reagents, Drugs, and Chemicals

Resveratrol (3,4',5-Trihydroxy-*trans*-stilbene) provided by **Sigma-Aldrich** (St. Louis, MO). SAHA (Vorinostat) was provided by BioVision (Mountain View, CA). LBH-589 (Panobinostat)

was provided by Novartis Pharmaceuticals Inc. (East Hanover, NJ). MnTBAP was provided by Calbiochem, CA. 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (ROS dye) was provided by Molecular Probes, Eugene, OR.

2.4 Experimental Format

Logarithmically growing cells were suspended in RPMI media containing 10% FBS (Atlanta Biologicals, Lawrenceville, GA) as described above. Cells were cultured at least 24 hours prior to treatment with designated drugs. For a specific incubation period as described below, after incubation, cells were transferred to sterile centrifuge tubes, pelleted by centrifugation at 1000 x g for 5 min at room temperature, and prepared for analysis as described below. MycoAlert Mycoplasma Detection Kit (*Lonza Rockland, Inc.*, ME) was used regularly to test for Mycoplasma contamination in all cell lines. All experiments used cells in logarithmic phase at 2.5×10^5 cells/mL

2.5 Assessment of Apoptosis

Apoptosis was evaluated by annexin V/propidium iodide (PI) (BD PharMingen, San Diego, CA) staining. Cells washed with 1x PBS. Cells then incubated with annexin V/PI for 30 min. at room temperature. Cells were then analyzed using a BD Biosciences FACScan flow cytometer.

2.6 Assessment of cell Viability

Cells were stained with 25 μ M 7AAD solution for 20 min. at 37°C in regular culture media and analyzed in the FL2 channel using the Becton Dickinson flow cytometer. Cells undergoing apoptosis were regularly monitored by Annexin V staining to confirm 7AAD results.

2.7 Assessment of Mitochondrial Membrane Potential

Cells were incubated with 40 nm DiOC₆ for 20 min at 37°C. Analysis was then carried out on a Becton Dickinson FACScan cytofluorometer. The percentage of cells exhibiting low levels of DiOC₆ is reflecting loss of mitochondrial membrane potential.

2.8 Measurement of ROS Production.

Cells were treated with 20 μ M 2',7'-dichlorodihydrofluorescein diacetate for 30 min. at 37°C, fluorescence was measured by flow cytometry on a FACS scan, and analyzed with CELL Quest software as described previously (46). MS-275 (Nihon Shering K. K. Pharmaceuticals, Chiba, Japan) was used as a positive control.

2.9 Western Blot Analysis

Whole cell pellets were washed twice in 1x PBS, resuspended in 1x PBS, and lysed by the addition of 1 volume of loading buffer. 30 μ g of total proteins per point were separated by 4–12%

Bis-Tris NuPage precast gel system (Invitrogen) and electroblotted to nitrocellulose. The blots were blocked in 5% nonfat milk in PBS-T and probed for 1 h with the appropriate dilution of primary antibody. Blots were washed 5×10 min in PBS-T and then incubated with a 1:2000 dilution of peroxidase-conjugated secondary antibody for 1 h at room temperature. Blots were again washed 5×10 min in PBS-T and then developed by enhanced chemiluminescence (New England Nuclear, Boston, MA). Where indicated, blots were stripped and re-probed with antibodies directed against actin. The primary antibodies and dilutions used were: histone H1.2 (1:3000; Abcam, Cambridge, MA), gamma-H2AX (1:2000; Upstate-Millipore, Billerica, MA), actin (1:4000; Sigma-Aldrich), caspase-8 (1:2000; Alexia Corp., San Diego, CA), acetylated Lys.310 (1:1000; Cell Signaling Technology), p21^{WAF1/CIP1} (1:1000; PharMingen-Transduction Laboratories, Lexington, KY), pro-caspase 3 (1:1000; PharMingen-Transduction Laboratories), cytochrome *c*, cyclin D1, cyclin A, cyclin E, and Mcl-1, DR4, DR5, caspase 9, p65 (1:1000; BD PharMingen),). cleaved caspase-3 and -9 (1:1000; Cell Signaling Technology, Beverly, MA); PARP (1:1000; BioMol, Plymouth Meeting, PA), apoptosis-inducing factor (AIF), Bid, pJNK, JNK2, SIRT1 (1:2000; Santa Cruz, Santa Cruz, CA). Secondary antibodies conjugated to horseradish peroxidase were from Kirkegaard and Perry Laboratories, Inc. (Gaithersburg, MD).

2.10 Analysis of Cytosolic Cytochrome *c* and AIF.

The S-100, or cytosolic fraction, was prepared as described in (141). Cells (20×10^6 /condition) were treated with the designated drugs concentrations and time. Cell then were harvested by centrifugation at 800 *g* for 10 min at 4°. The cell pellets were washed twice with ice-cold PBS and resuspended with 5 vol. of buffer A [20 mM HEPES–KOH (pH 7.5), 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 0.1 mM phenylmethylsulfonyl fluoride] containing 250 mM sucrose. The cells were incubated on ice for 30 min, and homogenized with a 25-gauge needle. The homogenates were centrifuged at 100,000 *g* for 30 min at 4°. The supernatants (S-100 fraction) were collected, and the protein concentration of S-100 was quantified using the Coomassie protein assay reagent. 30 µg of the S-100 fraction was loaded on the gel and probed with the corresponding antibody.

2.11 Immunoprecipitation Assay

Analyses of acetylated p65 protein by immunoprecipitation were performed as described (32). Cells were harvested (20×10^6 /condition), washed in ice-cold PBS, suspended in immunoprecipitation assay CHAPS lysis buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS in PBS) containing protease inhibitors, and quickly sonicated on ice. After centrifugation, the supernatant was incubated with antibody against p65 (BD Transduction Laboratories) and rocked overnight at 4°C. Immune complexes were immunoprecipitated with immunomagnetic microspheres, Dynabeads M-450 precoated with sheep anti-Mouse IgG (Invitrogen). The beads were washed with radioimmunoprecipitation assay buffer, treated with 1× sample buffer, boiled, and loaded in precast gels for Western blot analysis using acetylated Lysine probe.

2.12 Cell Cycle Analysis

4x10⁶ million cells / sample were treated with the designated drugs concentrations, after drug treatment, cells were pelleted by centrifugation at $500 \times g$ for 6 min and resuspended in 70% ethanol. The cells were incubated on ice for at least 1 h, resuspended in 1 ml of cell cycle buffer (0.38 mM sodium citrate, 0.5 mg/ml RNase A, and 0.01 mg/ml PI) at a concentration of 10×10^5 cells/ml, and stored in the dark at 4°C until analysis (24 h), using a Becton Dickinson FACScan flow cytometer and ModFit LT 2.0 software (Verity Software House, Topsham, ME).

2.13 DNA synthesis and S phase analysis

S phase was determined by incorporation of the thymidine analogue 5-ethynyl 2-deoxyuridine into genomic DNA using Click-iT™ EdU CellCycle 488-red (7-AAD) Flow Cytometry assay kit (Invitrogen). Cells were treated with the designated agent, labeled with 10 μM, EdU 2 hr prior to harvesting, cells then processed according to the manufacturer instructions, and stained for DNA content with CellCycle 488-red (7-AAD) 2 μL for 30 min to determine DNA content. Cells were analyzed by the Becton Dickinson flow cytometer using CellQuest software. The content of S phase cells was evaluated by determining percentage of EdU-FITC positive populations.

2.14 NF-κB Activity

Nuclear protein was extracted using Nuclear Extract Kit (Active Motif). NF-κB activity was determined using an enzyme linked immunosorbent assay (ELISA) Kit TransAM NF-κB p65 Transcription Factor Assay Kit (Active Motif), according to the manufacturer's instructions and as described (142). Briefly, the activated form of NF-κB that is present in nuclear extracts was detected by using an anti-p65 specific antibody that recognizes the NF-κB bound to a consensus DNA oligonucleotide immobilized in a 96-well plate. Addition of a secondary antibody conjugated to horseradish peroxidase provides sensitive colorimetric readout by spectrophotometry.

2.15 Statistical Analysis

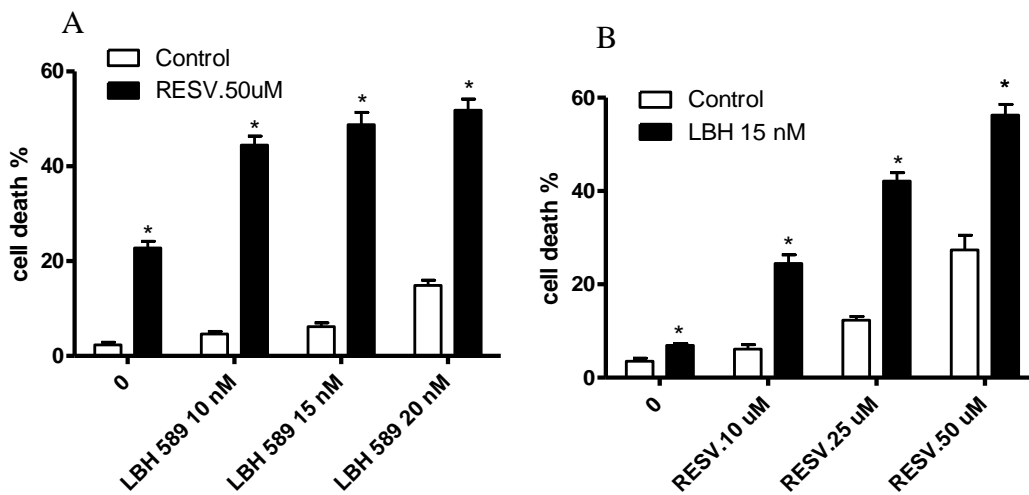
The significance of differences between experimental conditions was determined using the two-tailed Student t test. Characterization of synergistic and antagonistic interactions was performed using Median Dose Effect analysis in conjunction with a commercially available software program (CalcuSyn, Biosoft, Ferguson, MO).

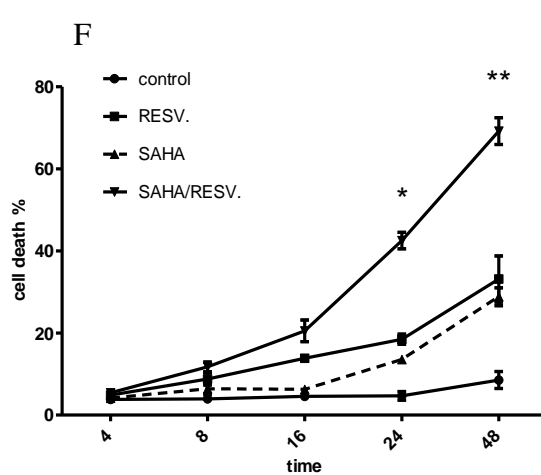
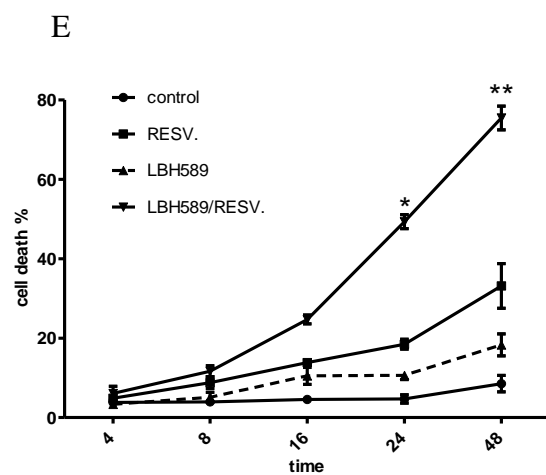
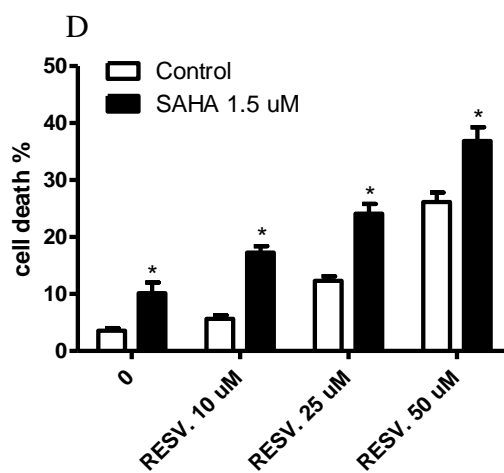
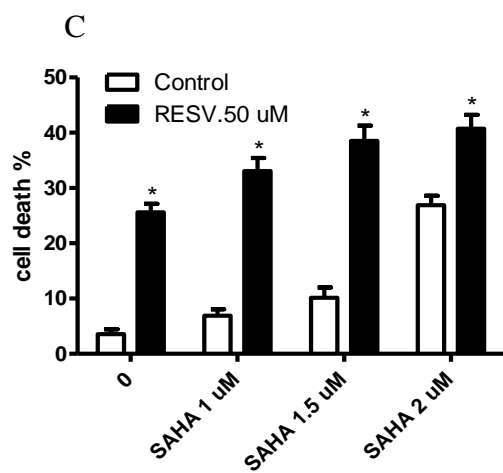
3. Results

3.1 Resveratrol interact synergistically with HDAC inhibitors in U937 cells to induced apoptosis:

To test whether the regimen combining Resveratrol with pan-HDACI Vorinostat (SAHA) or LBH-589 (Panobinostat) promote apoptotic cell death in U937 myelomonocytic leukemia cells, we exposed U937 cell to simultaneous treatment with Resveratrol and HDAC inhibitors (Vorinostat-SAHA or Panobinostat-LBH-589) for multiple concentrations of each drug, then we measured percentage cell death by analyzing 7AAD stain uptake at 24hrs. We found that at different concentrations there is significant increase in cell death when comparing each combination to its respective single drug treatment (Fig-1 A, D). A similar phenomenon was found when we compared the combination treatment to each single agent in that combination at different time points (Figure 1E, F). These results were confirmed by staining U937 cell with Annexin V/PI. (Data are not shown). To test for synergistic interactions between Resveratrol and HDACIs using Median Dose effect analysis we found that combined RESV-HDACI exposure for 24 hours resulted in a synergistic increase in cell death with CI values less than 1.0 (Figure 1G, J). MV4-11 cells treated with this combination showed similar finding (data not shown).

Fig.1





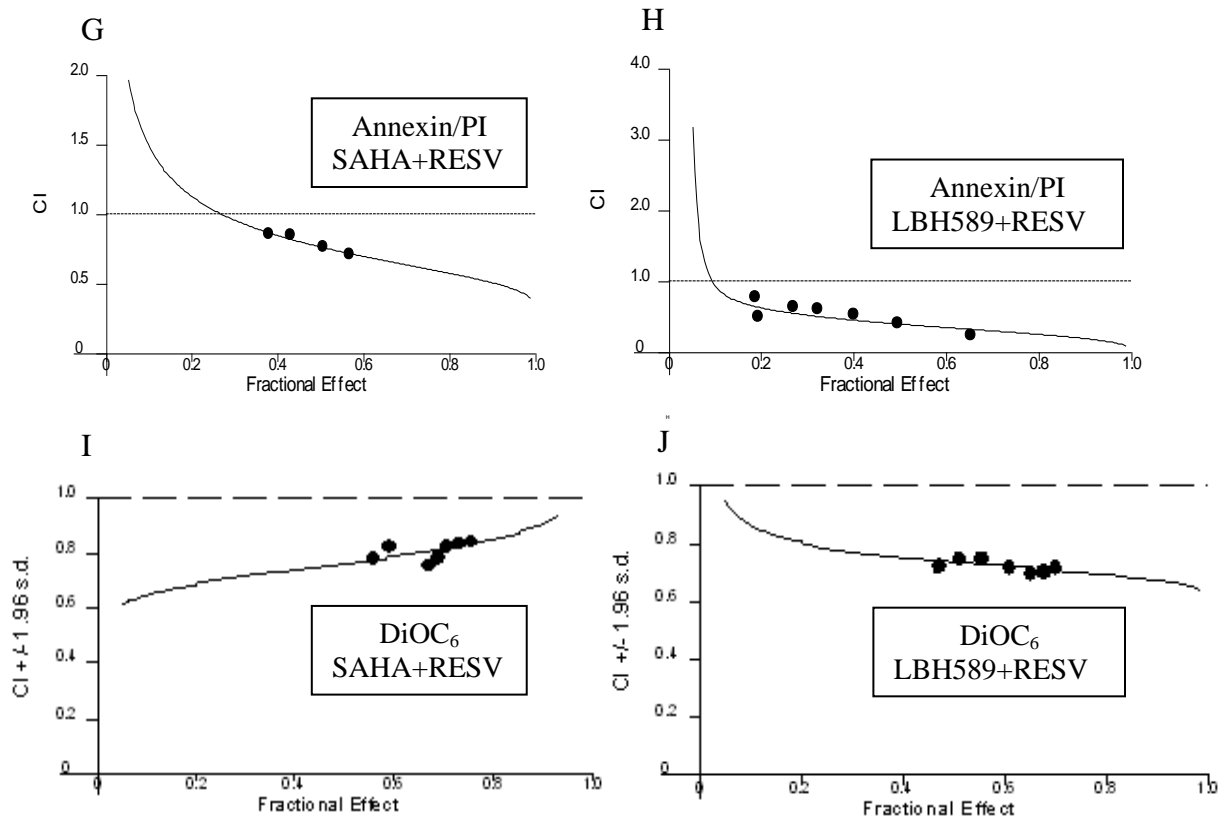


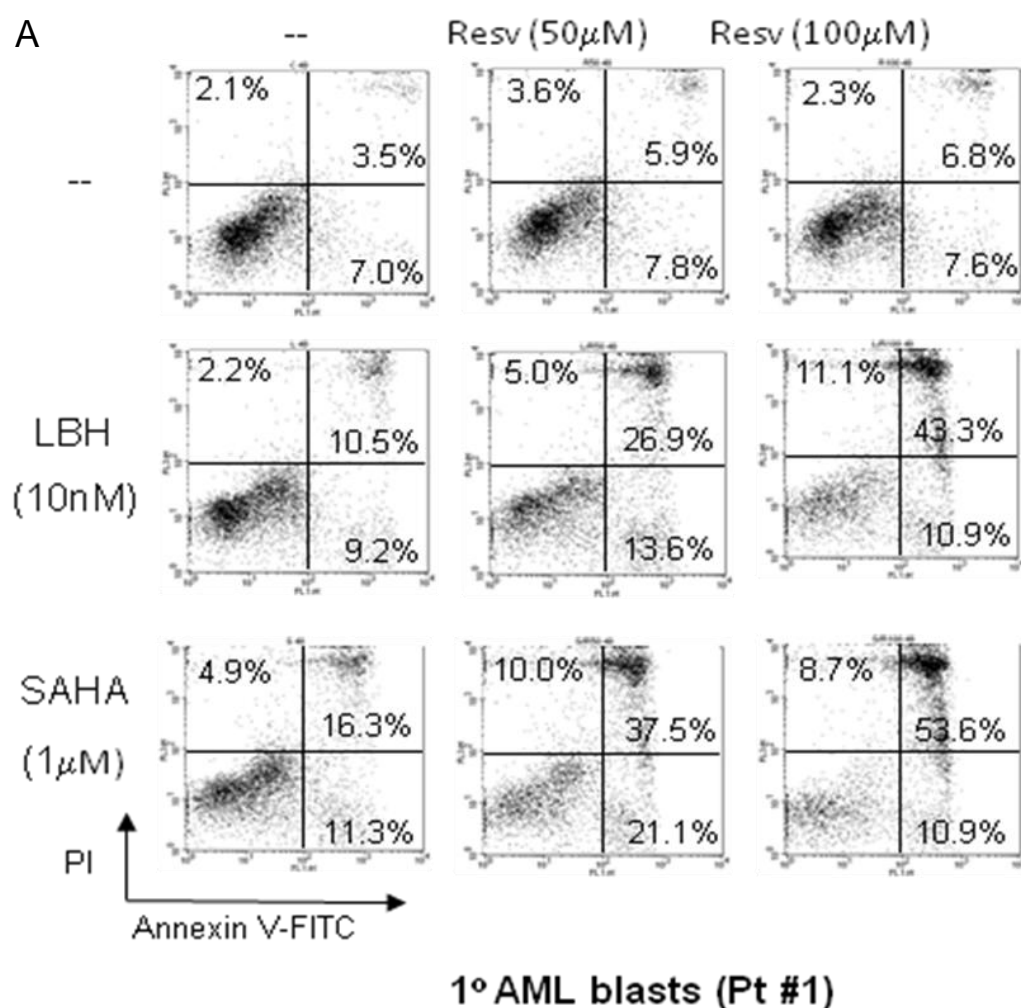
Figure1 *Resveratrol interacts synergistically with HDAC inhibitors in U937 cells to induce apoptosis.*

(A-D). U937 cells we treated with increasing concentrations of Vorinostat (0.1-2.0uM), LBH-589 (10-20 nM) with RESV (10-50 uM) for 24hrs, after which cell death was monitored by 7AAD/DiOC₆ double staining. Values represent mean \pm S.E., * $p < 0.05$ (E-F). U937 cells we treated with vorinostat SAHA (1.5 uM), or LBH-589 (15 nM) for 4-48 hr. after which cell death was monitored by 7AAD/DiOC₆ double staining. Results Values represent mean \pm S.E., * $p < 0.05$, ** $p < 0.01$ (G-J) Fractional Effect values were determined by comparing apoptotic cell death of RESV-HDACI to those of untreated or single drug-treated controls, and Median Dose Effect analysis was employed to characterize the nature of the interaction. Combination Index (C.I.) values less than 1.0 denote a synergistic interaction. Apoptosis was determined by using Annexin/PI staining (G-H). or loss of mitochondrial membrane potential using DiOC₆ stain (I-J). G and I: SAHA+RESV. H and J: LBH589+RESV.

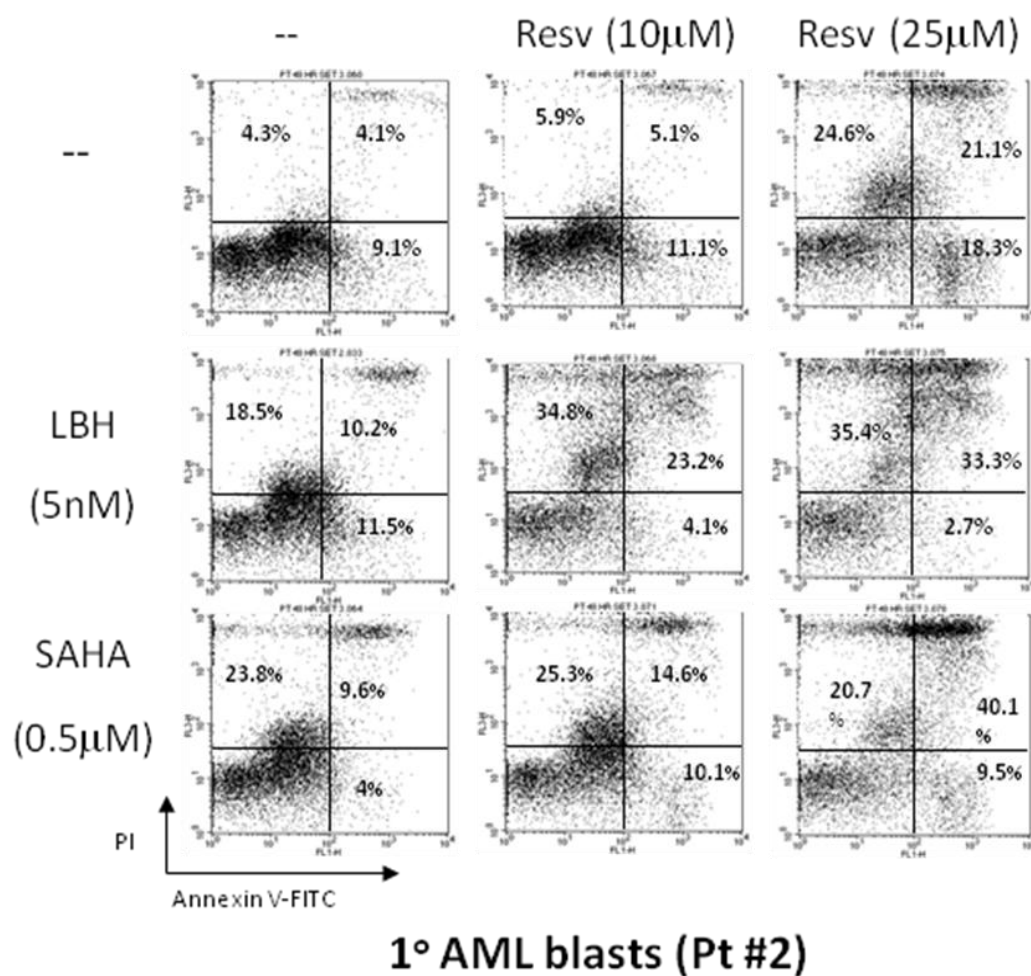
3.2 Regime combination RESV+HDACIs is active in Primary blasts cell but not cord CD34+ cells.

To verify whether our findings of synergistic interaction of Resveratrol and LBH589/SAHA can be re-capitulated in primary cells, we assess this interaction in primary AML samples. Single drug exposure for 24 hr to 50-100 μ M of Resveratrol and low concentrations of LBH589/SAHA in patient #1 (fig2A) induce limited apoptotic cell death, while the combination of RESV-SAHA/LBH589 increase lethality to significant level. Another, primary AML sample (Patient #2 fig2B.) was more sensitive to lower concentrations of Resveratrol and HDACIs, yet we observed marked increase (include values) in cell lethality with the drug combination. To test whether this combination is toxic to normal cells, CD34+ cells isolated from cord blood and treated with Resveratrol (25 μ M) LBH589 (10nM), SAHA (1 μ M) or a combination of RESV-SAHA/LBH589 for 24hrs was analyzed using Annexin V/PI staining. Cord blood sample showed limited lethality in single drug exposure with minimal increase in lethality in RESV-HDACI combination (Fig2C.) implying no significant interaction of this combination treatment in normal cord blood cells.

Fig.2



B



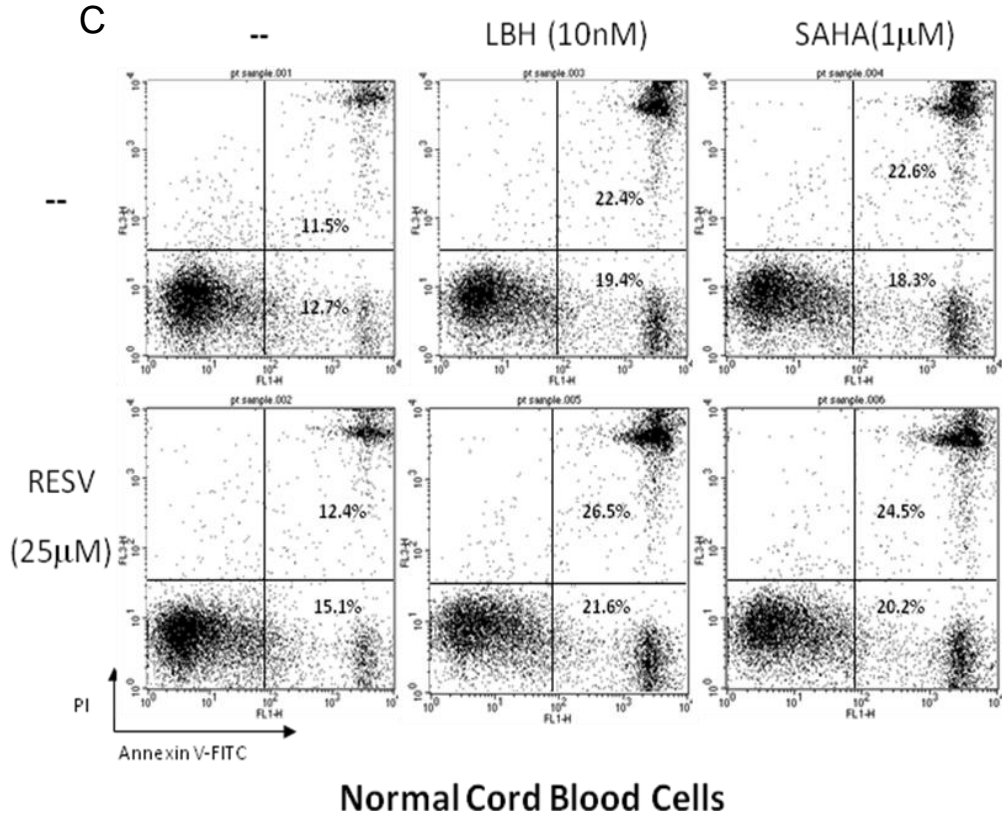


Figure 2: Regime combination RESV+HDAC I is active in Primary blasts cell but not cord CD34+ cells.

(A-B) Primary cells isolated from patients with AML, exposed to the designated concentrations of Resveratrol, LBH589, and SAHA and assisted for the level of cell death. The percentage of apoptotic cells was determined by Annexin V/PI staining and flow cytometry, 10,000 events/sample. (C) Normal CD34+ cord blood cells exposed to the same Drugs. Apoptosis were determined by Annexin V/PI staining and flow cytometry, 10,000 events/sample.

3.3 RESV-HDACI Co-treatment induces activation of caspase cascade (especially casp8) in association with activating mitochondrial-dependent pathways and increase DNA damages.

HDACI induce Leukemic cell death by different mechanisms including activation of caspase cascades and induction of mitochondrial injury as discussed in above. Caspase-8, and FADD activation have roles in promoting the cytotoxicity and increased superoxide levels in leukemic cell (143). We want to explore the cellular mechanisms by which RESV-HDACI co-administration induce apoptotic cell death in U937. Western blot analysis of cytosolic fraction (S100) of U937 after treatment with RESV, HDACIs or a combination of both revealed a significant increase in the amount of mitochondrial proteins, cytochrome *c* released from the mitochondria to the cytosol in RESV-SAHA/LBH589 compared to single drug treatment. Apoptosis Inducing Factor (AIF) release to cytosol was markedly increased in RESV-SAHA treatment compared to SAHA alone (fig3 A). The mitochondrial membrane potential ($\Delta\psi_m$) was determined in U937 after exposure to the designated treatment RESV-SAHA/LBH589 resulted in a synergistic increase in loss of mitochondrial membrane potential $\Delta\psi_m$ compared to single drug treatment fig.1 (I-J). Moreover, there is a marked increase in the DNA damage marker γ H2AX in RESV-HDACI co-administration compared to single drug administration (fig3-A). We further explore the role of Extrinsic and intrinsic pathways activation in RESV-HDACI treatment. Interestingly, we found a significant increase in the extrinsic caspase pathway activation represented by increase casp8 cleavage in RESV-SAHA/LBH589 compared to RESV or HDACIs alone. casp3 also showed marked cleavage in combination treatment, while casp9 activation shows moderate increase if compared to single RESV or HDACI treatment alone (Fig3-C).

Fig.3

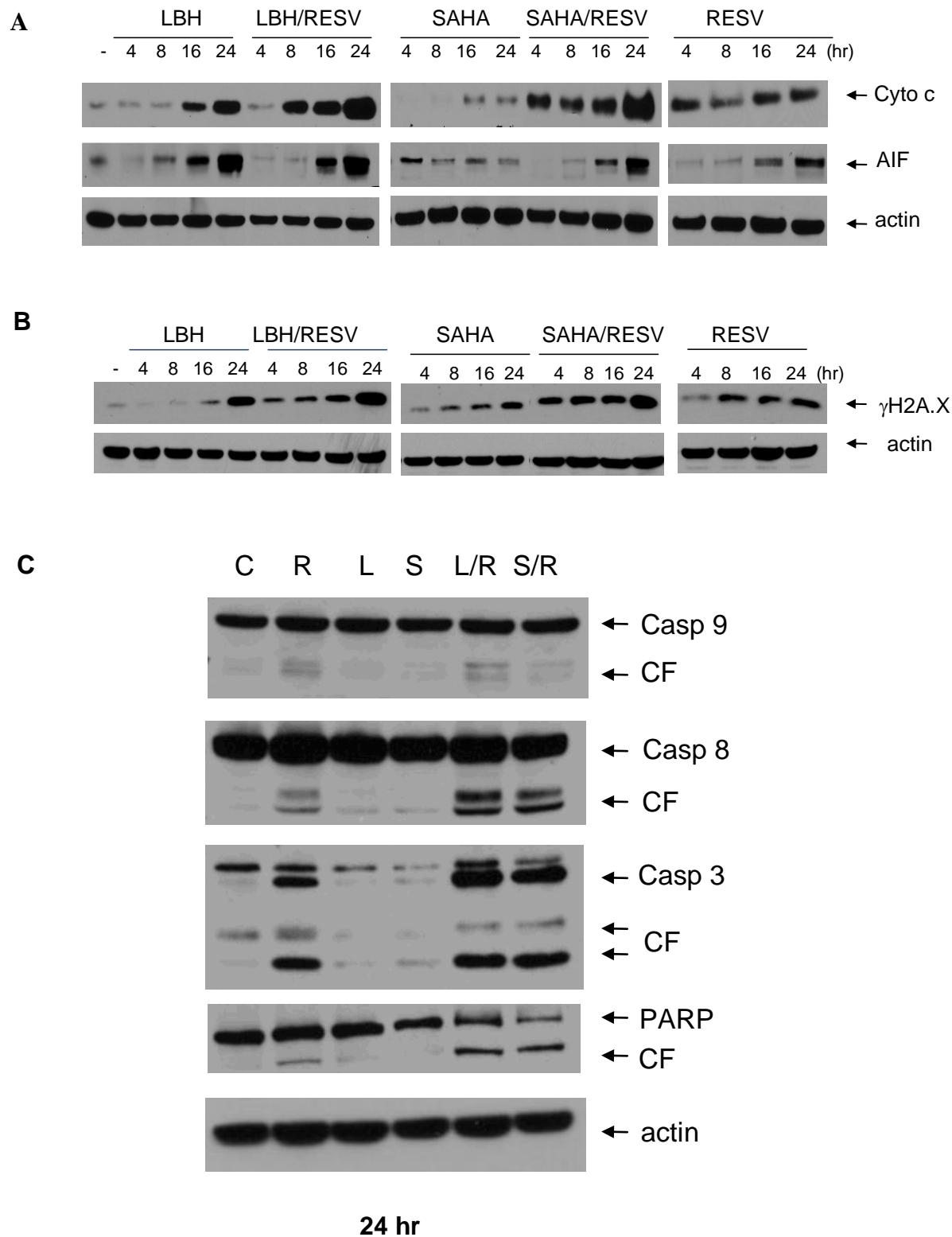


Fig3 *RESV-HDACI Co-treatment induces activation of caspase cascade (especially casp8) in association with activating mitochondrial-dependent pathways and increase DNA damages.*

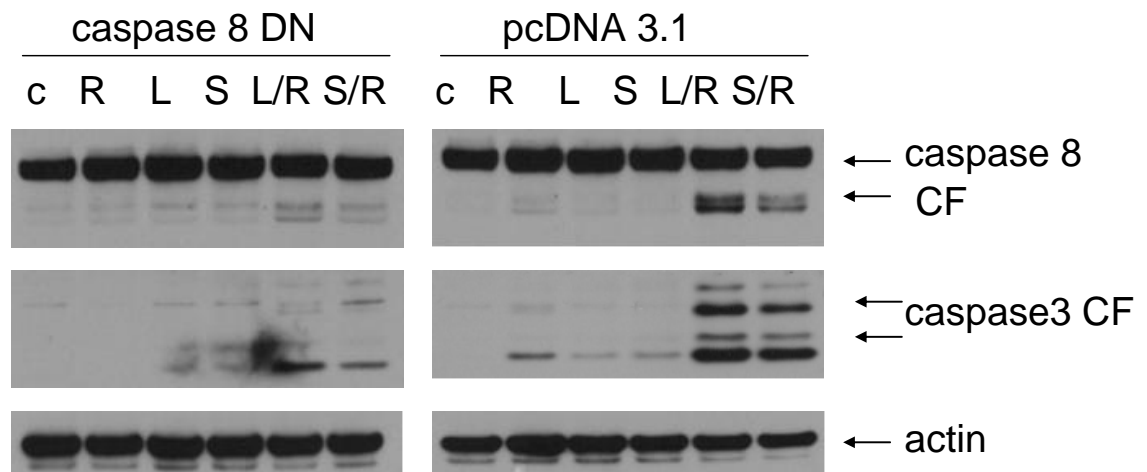
(A) U937 Cells were treated with either LBH589 (15 nM), SAHA (1.5 μ M), \pm Resveratrol (50 μ M) for 4-24 hrs. Then we run Western blot analysis on cytosolic fraction (S100) with the indicated primary antibodies markers of mitochondrial injury. Blots were then stripped and reprobed with anti-actin to ensure equal loading and transfer of protein (30 μ g in each lane). (B-C) Western blot analysis of U937 cells lysate exposed to LBH589 (15 nM), SAHA (1.5 μ M), \pm Resveratrol (50 μ M) for 4-24 hrs for marker of DNA damage (B) and 24 hrs for caspase activation marker (C) actin marker was used to ensure equal loading of protein. C: control, R: Resveratrol, L: LBH589, S: SAHA.

3.4 Functional role of extrinsic pathway in promoting RESV- HDACI U937 Cell death.

In the light of previous results, we further sought to explore the role of the extrinsic pathway in regulating U937 cell death. U937 casp8 Dominant negative cell (casp8 DN) and their pcDNA 3.1 control counterpart were exposed to HDACIs either LBH589 (15 nM), or SAHA (1.5uM), and Resveratrol (50 uM) or a combination of both, cleavage of casp8 is lost in casp8 DN cells but not the control cells as was expected, see (Fig4A). Markers of cleaved casp3 showed blocking of casp3 activation in casp8 DN. Then, we monitored the cell viability by measuring the uptake of 7AAD in casp8 DN clone and compare the results to the pcDNA 3.1 control under the same condition. We found that blocking casp8 activity significantly protected U937 cells and decreased cell death by 30-40% in casp8 DN clone when exposed to a combination of RESV-HDACI compared to pcDNA 3.1 control. (Fig4B).

Fig.4

A



B

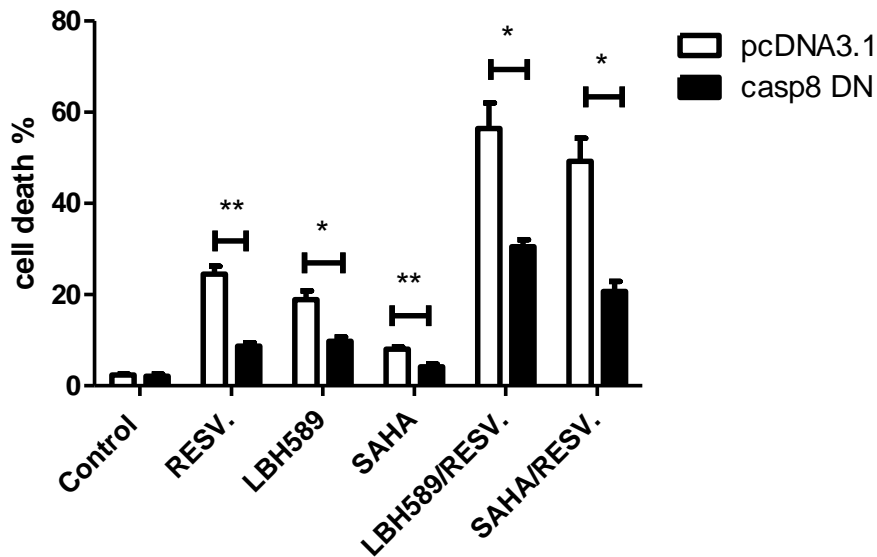


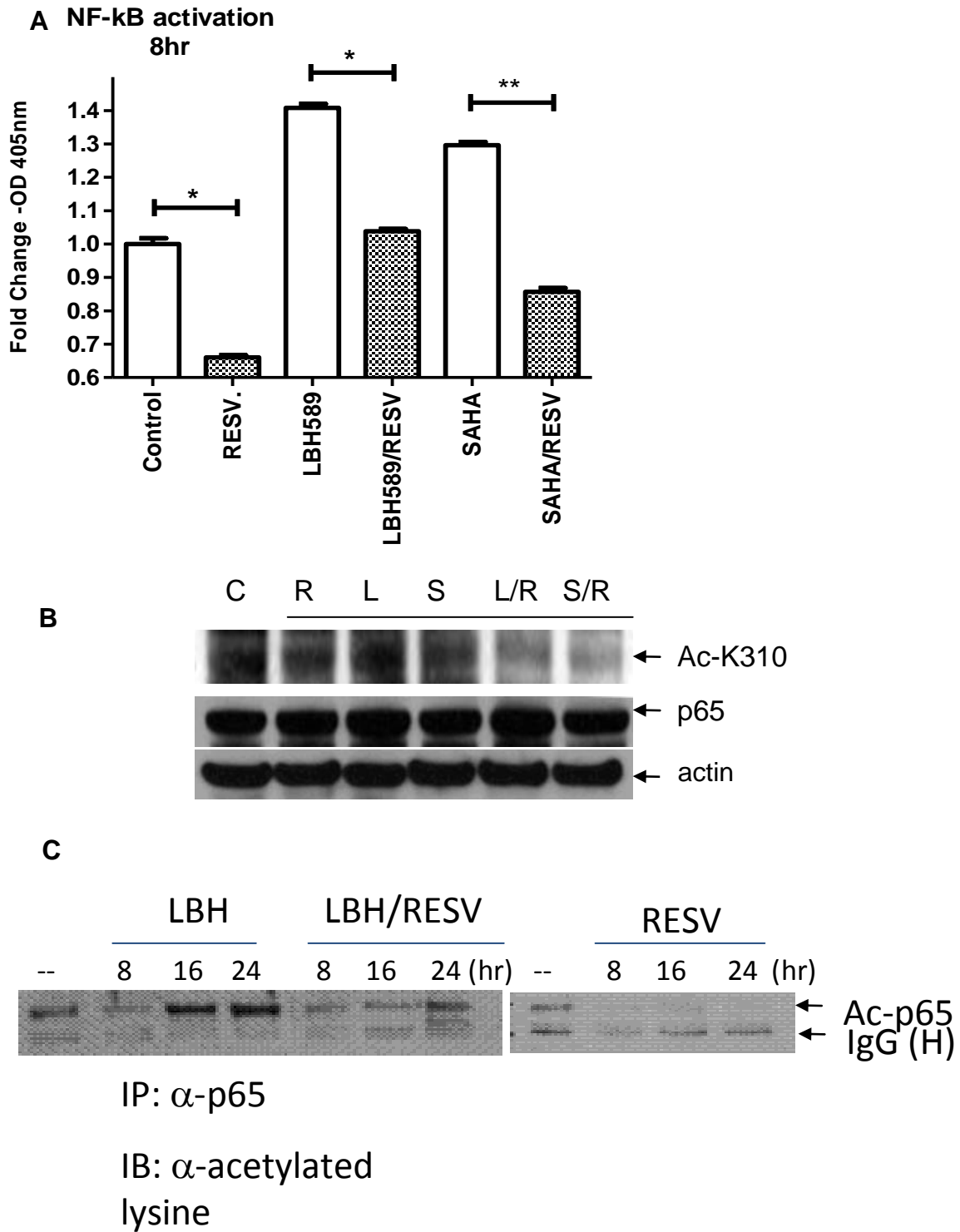
Fig4. Functional role of extrinsic pathway in promoting RESV- HDACI U937 Cell death.

(A) Western blot analyses of casp8 and cleaved casp3 in whole cell lysates of U937 casp8 Dominant negative cell (casp8 DN) and their pcDNA 3.1 control counterpart after exposure to LBH589 (15 nM), SAHA (1.5uM)± Resveratrol (50 uM) as indicated. Actin was used to ensure equivalent loading and transfer. (B) U937 casp8 Dominant negative cell (casp8 DN) and their pcDNA 3.1 control counterpart cells exposed to LBH589 (15 nM), or SAHA (1.5uM) ± Resveratrol (50 uM) for 24 hrs. Cell death was determined by flow cytometry analysis of 7AAD-positive (%) cells. Values represent mean ± S.E.*p<0.05, **p<0.01.

3.5 Resveratrol blocks NFκB activation induced by HDACI through activation of SIRT-1. Yet SIRT1 knockdown failed to protect U937 cells against HDACI lethality.

It was previously reported that Resveratrol activates SIRT1 HDAC. SIRT1 blocks the HDAC inhibitors induced NF-κB activation via deacetylation (120-122); we then sought to test the hypothesis that NF-κB deacetylation by SIRT1 after Resveratrol exposure in leukemia cell line and primary blood cells is responsible for RESV+HDACIs synergistic interaction. U937 cell were exposed to minimally toxic concentration of Resveratrol ± LBH589 and then nuclear protein was extracted using Nuclear Extract Kit (Active Motif). NF-κB activity was determined using an enzyme linked immunosorbent assay (ELISA). NF-κB activity was increased as was expected after HDACIs exposure, but the combination treatment caused significant decrease in NF-κB activity within 8 hrs after treatment (Fig5A). Western blots analysis using a marker for acetylated p65 (Lys310) showed a marked deacetylation of K310 after 24 hr of RESV+HDACI compared to HDACI alone (fig5. B). similar results of NF-κB deactivation in combination treatment found when we immunoprecipitated p65 with an anti-Acetylated Lysine antibody. These findings associated with HDACIs mediated induction of p21 which is at least partially regulated by NF-κB (25). Interestingly, Resveratrol blocked p21 induction in the combination (data not shown). Depending on above, we want to assess the role of SIRT1 activation by Resveratrol in promoting RESV+HDACIs interaction. We knocked down SIRT1 by transfecting U937 cells with small non-specific interfering RNA (siRNA) and we established a shSIRT cell lines, we selected two cell lines with lower SIRT1 expression (Fig5D) shSIRT1 clones showed increased acetylation of p65 due to loss of SIRT1 function we then expose these two clones and their control counterpart (U937 shNC-A5) to HDACIs and we assessed cell death after 24 hrs using 7AAD staining. We did not observe a significant difference in cell death after knocking down SIRT1 in U937 using minimum toxic doses of SAHA (1.5 uM) or LBH589 (15 nM) (Fig5D; P > 0.05). Moreover, U937 exposed to higher doses of HDACIs showed increased cell death in shSIRT1 clone more than control (data not shown). These results suggest that SIRT1 does not clearly have a significant role in inducing cell death observed in the RESV+HDACIs combination.

Fig.5



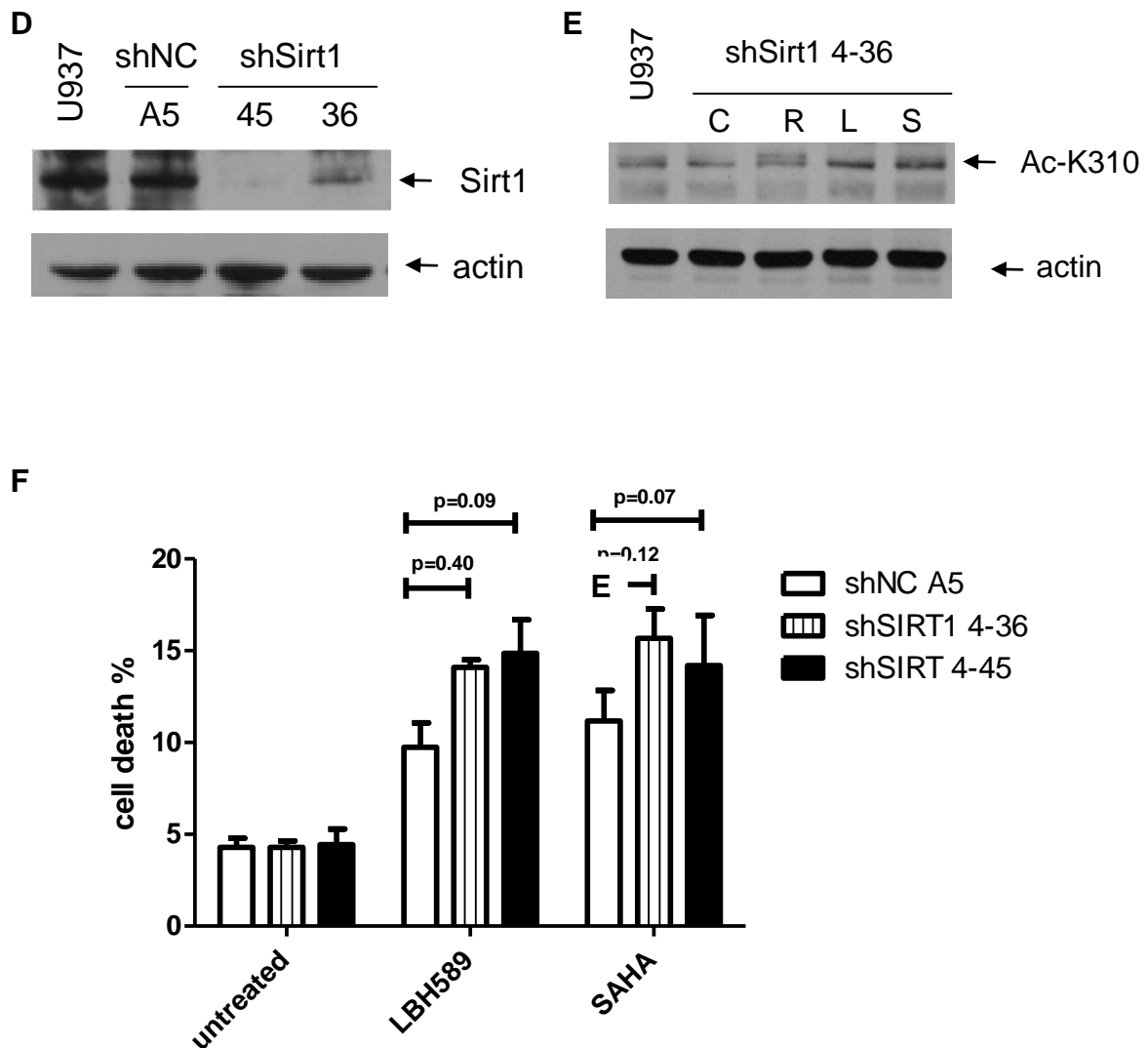


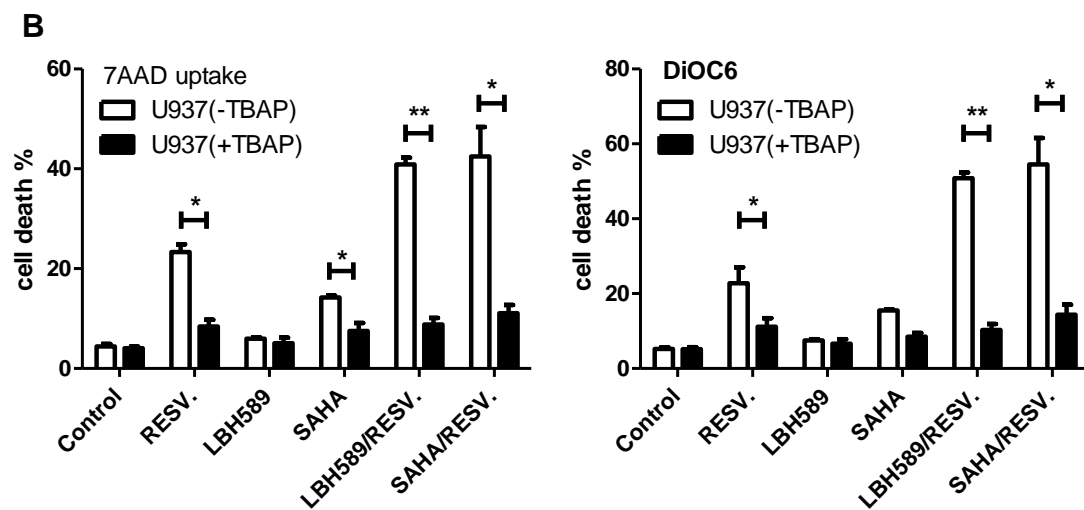
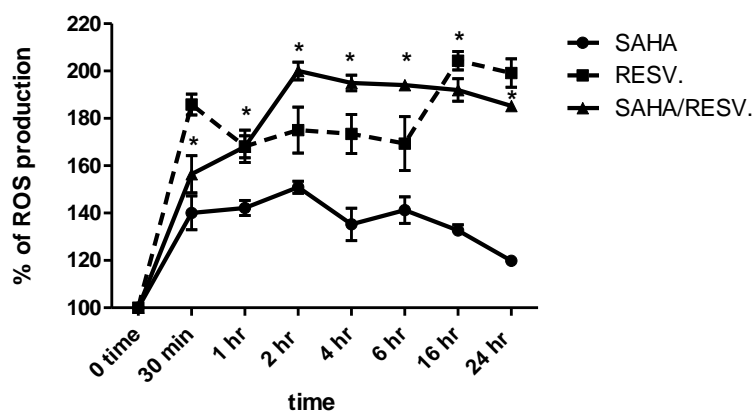
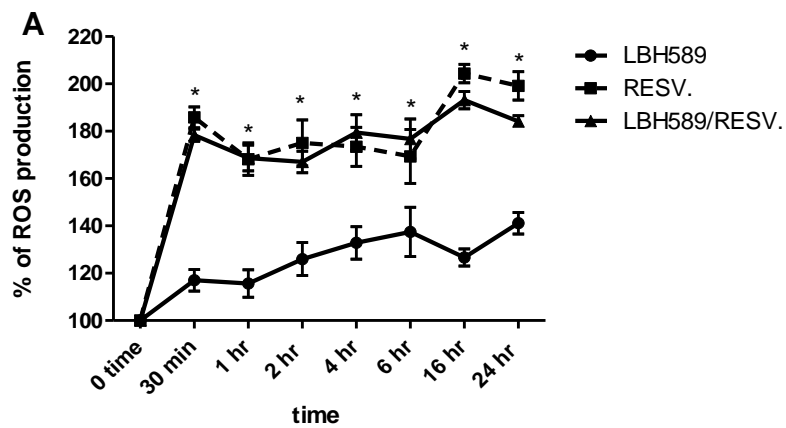
Fig5 Resveratrol blocks NFκB activation induced by HDACI through activation of SIRT-1. Yet SIRT1 knockdown failed to protect U937 cells against HDACI lethality.

(A) NF-κB Activity: the activated form of NF-κB that is present in U937 nuclear extracts after 8 hrs exposure to the respective drug/s was detected by using an anti-p65 specific antibody that recognizes the NF-κB using an enzyme linked immunosorbent assay (ELISA). Values represent mean ± S.E. *p<0.001, **p<0.00001. (B) Western blot analysis on U937 Cells lysate after treatment with LBH589 (15 nM), SAHA (1.5uM) ± Resveratrol (50 uM) for 24 hrs. Blots were then probed for marker of deacetylated K310, p65, and actin to ensure equal loading and transfer of proteins. (C) Lysates from U937 cells after treatment with LBH589 (15 nM), SAHA (1.5uM) ± Resveratrol (50 uM) (at indicated intervals) were immunoprecipitated (IP) with an anti-p65 antibody followed by Western blot (WB) analysis with an anti-Acetylated Lysine antibody. IgG bands are shown as loading controls.(D) Western blot analyses of SIRT1 in whole cell lysates of U937, shNC- A5, and two shSIRT1 cell lines. Actin was used to ensure equivalent loading and transfer. (E) Western blot analysis on U937 and shSIRT1 4-36 clone Cells lysate after treatment with LBH589 (15 nM), SAHA (1.5uM) and Resveratrol (50 uM) for 24 hrs. Blots were then probed for marker of deacetylated K310, p65, and actin to ensure equal loading and transfer of proteins. (F) Cell death induction was determined by flow cytometry analysis of 7AAD-positive (%) U937 cells ± LBH-589 or SAHA (15 nM 1.5 uM respectively for 24 h). Values represent mean ± S.E.

3.6 ROS (reactive oxygen species) plays a critical functional role in interaction between RESV+HDAC I:

HDACIs are known to induce ROS generation (25, 42). On the other hand, Resveratrol and other Polyphenol can act as an antioxidant or a pro-oxidant depending on cell type, dose, and duration of exposure as discussed previously(113). We studied whether RESV+HDACIs combination induced lethality involves ROS induction to a higher level if compared to HDACIs exposure alone. Exposure of U937 cell to LBH589 (15 nM), or SAHA (1.5uM) induce ROS generation while a combination of one of the above drugs with Resveratrol (50 uM) significantly increase ROS generation. ROS generation in U937 treated with Resveratrol (50 uM) alone show insignificant difference than the combination treatment except that there was increase in ROS induction in early time of SAHA/RESV exposure (2-6 hrs) compared to what is observed with Resveratrol exposure alone (Fig.6A). Significantly, the enhanced lethality observed in RESV+HDACIs exposure was abrogated after co-incubation of cells with the ROS scavenger (Mn-TBAP), which blocked ROS production(25), attenuated the pronounced RESV+HDACIs-mediated apoptosis and mitochondrial injury (as measured by 7AAD and DiOC₆ stains) observed in U937 cells (Fig6 B). Co-administration of Mn-TBAP also blocks caspase 8 and caspase 3 activation as well as PARP cleavage. (Fig6 C)

Fig.6



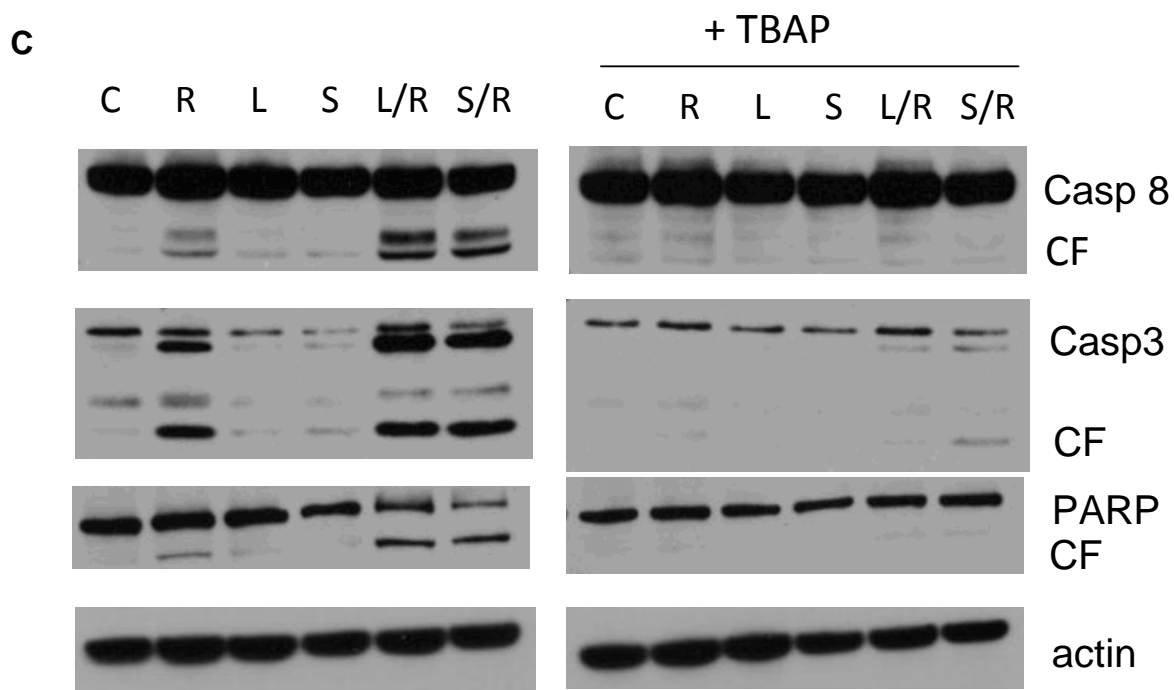


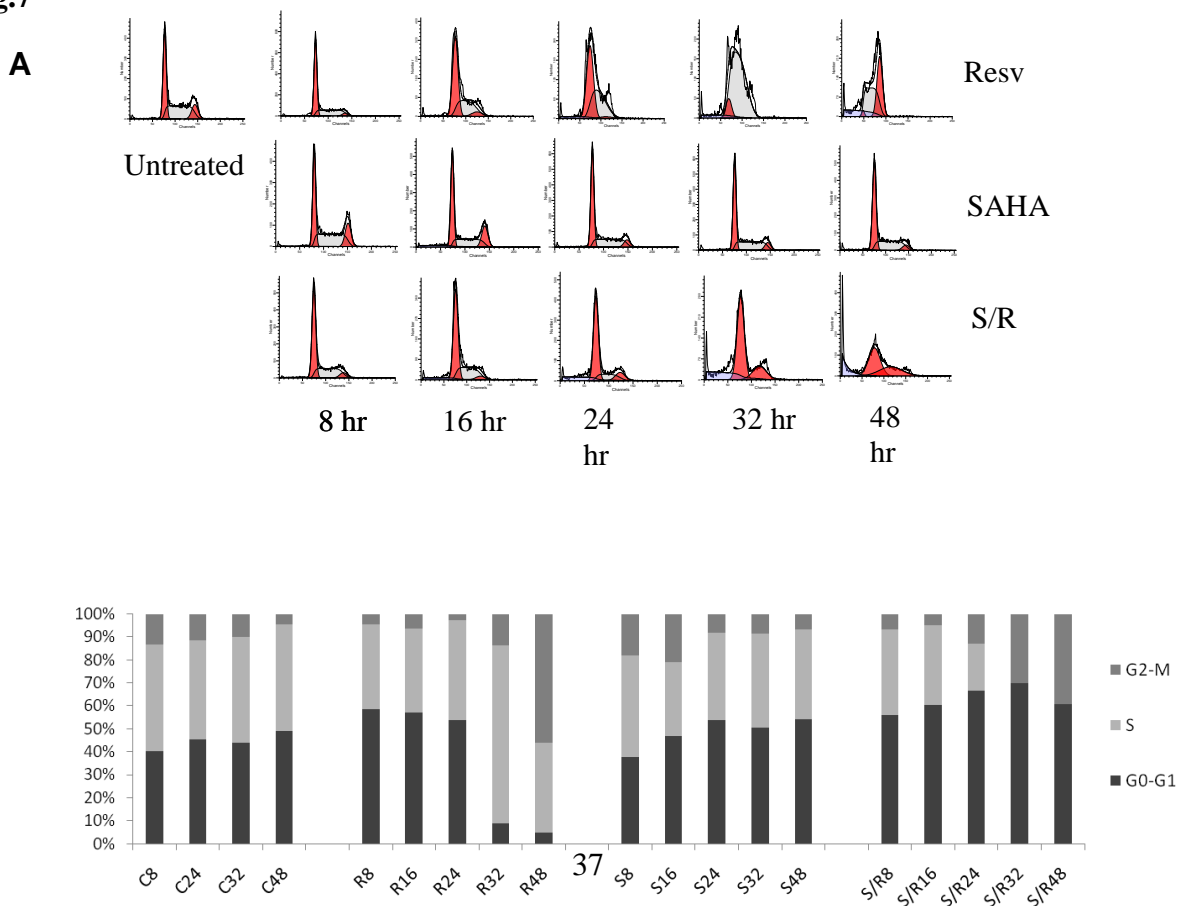
Fig 6 *ROS (reactive oxygen species) plays a critical functional role in interaction between RESV+HDACIs*

(A) Levels of ROS quantified in U937 after exposure to LBH589 (15 nM), or SAHA (1.5uM) \pm Resveratrol (50 uM) (at indicated intervals). Cells were labeled with the oxidation-sensitive dye H₂DCFDA (20 μ M) and analyzed by flow cytometry. Values represent mean \pm S.E. (B) U937 cells exposed to LBH589 (15 nM), or SAHA (1.5uM) \pm Resveratrol (50 uM) for 24 hrs. Cell death induction was determined by flow cytometry analysis of 7AAD-positive (%) U937 cells and mitochondrial membrane potential using DiOC₆ stain. Values represent mean \pm S.E. (C) Western blot analyses of caspase8, caspase3 and PARP in whole cell lysates of U937 after exposure to LBH589 (15 nM), SAHA (1.5uM) \pm Resveratrol (50 uM) as indicated with or without co-incubation of cells with Mn-TBAP (400 μ M). Actin was used to ensure equivalent loading and transfer. *p<0.05, **p<0.01.

3.7 Resveratrol induces S Phase arrest which abrogated by HDAC I co-administration leading to more cell death.

Resveratrol, as well as HDACIs are known to cause perturbations in cell cycle (69, 137). To this extent we want to test whether RESV+HDACIs co-administration has a role in causing U937 cell cycle arrest, we analyzed cell cycle distribution in U937 cell after exposure to SAHA (1.5uM), Resveratrol (50 uM) or the combination at different durations (16-48 hrs). Results indicate marked increase in G0-G1 phase of cell cycle after 24 hr of Resveratrol treatment followed by dramatic increase in percentage of S phase at 32 hrs. Regarding SAHA, there was a minimal to moderate change in cell cycle. However, the combination of both drugs induces G0-G1 arrest especially at 24-48 hrs after exposure (Fig7A).) To assess DNA synthesis, we measured the change in S phase distribution using Click-iT™ EdU Flow Cytometry Assay Kits at different durations (16-48 hrs) after exposure to SAHA (1.5uM) ± Resveratrol (50 uM). The results indicate a marked block of DNA synthesis in RESV+SAHA combination compared to Resveratrol or SAHA treatment alone at 24-48 hrs. (Fig.7 B). Similar results were also reported in case of LBH589/RESV+LBH589 treatment (data not shown).

Fig.7



B

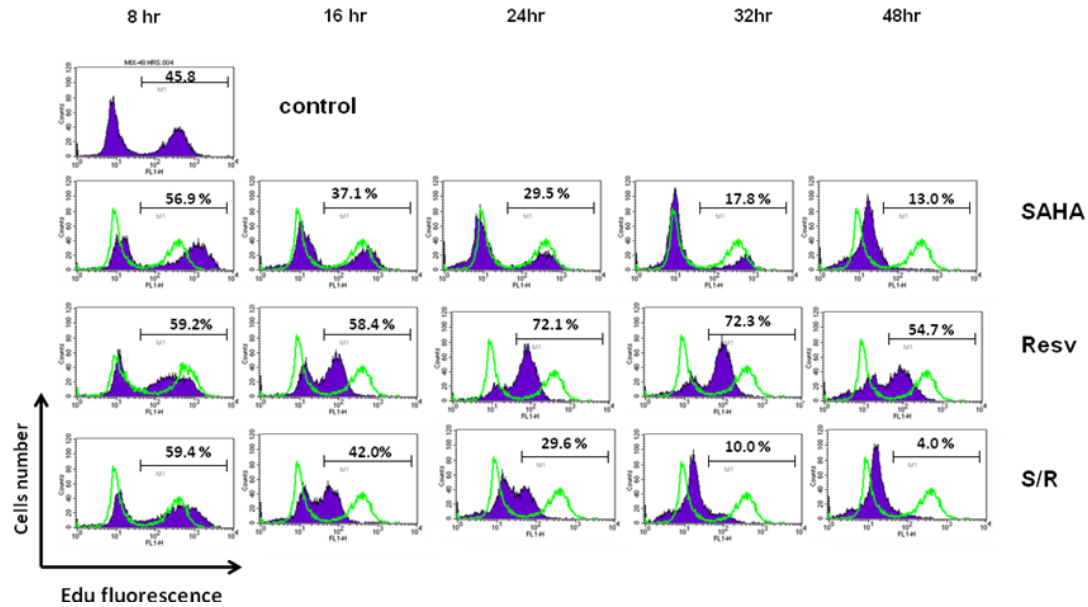


Fig7 Resveratrol induces S Phase arrest which abrogated by HDAC I co-administration leading to more cell death.

(A) U937 cells were treated with SAHA (1.5uM), Resveratrol (50 uM) or both, fixed with 70% ethanol and analyzed for cell cycle distribution using flow cytometry as described in methods. Lower panel is quantification to the cell cycle distribution. (B) U937 cells were treated with SAHA (1.5uM), Resveratrol (50 uM) or a combination of both, Incubate with Click-iT™ EdU for 2 hrs, premobized and then Analyzed for DNA synthesis by flow cytometry as described in methods.

4. Discussion

Many HDAC inhibitors have been tested in clinical studies, for instance SAHA was approved by the FDA in 2006 for treatment of cutaneous manifestations of cutaneous T-cell lymphoma(76). Other HDAC Inhibitors are also evaluated recently in clinical trials for treatment of cancers including many hematologic malignancies (79). Over the last several years multiple mechanisms of HDACI induced lethality in leukemia has been identified as discussed previously. The resistance of leukemic cells to HDACI exposure depends on different factors, one of which is perturbations in the survival signaling pathways status, such as NF- κ B(55) For example, HDACIs can induce initial (70) and sustained NF- κ B activation through different mechanisms e.g. RelA acetylation(71). HDACI-mediated perturbations in NF- κ B play a central role in attenuation of drug lethality (55). Thus, RelA/p65 acetylation or other mechanisms responsible for sustained activation of the NF- κ B axis may be responsible for limiting the apoptotic cell death induced by HDACIs exposure. One of the major challenges that limit the successful HDACI development in clinical application is agent's toxicity especially the cardiac side effect (80), other HDACI acute toxicities include general fatigue, gastrointestinal symptoms, and transient cytopenias (77). To overcome these challenges many drug combination had been suggested in order to use minimal toxic doses of HDACIs and overcome the protective cellular responses that the cancerous cell develop. SIRT1 is a HDAC class III enzyme which has been shown to mediate deactivation of NF- κ B through deacetylation (122). However, Sirtuins are also involved in many other physiological and pathological processes, targeting many cellular and metabolic responses (12). Resveratrol is a Polyphenol compound found in grapes, red wines, berries, and peanuts (82). Many studies provided abundant evidences for the use of Resveratrol as a chemopreventative agent and the potential for its clinical use in preventing and treating cancer (105). Notably, Resveratrol is known to act as an activator of the SIRT1 HDAC (120, 144).

The central goal of this regimen was to develop a rational basis for enhancing the antileukemic activity of HDACIs by combining them with agents that target critical survival signaling pathways. Thus, a combination of HDACIs and NF- κ B inhibitors SIRT1 agonists such as Resveratrol would result in enhanced lethality of leukemic cells. As long as we know, this study is the first study that uses Resveratrol and HDACIs combination for cancer treatment.

In this study there was potentiation of cell death in human U937 leukemia cell lines and patient samples exposed to combinations of Resveratrol and HDACIs. The interaction in this treatment regimen was synergistic using minimally toxic concentrations of both drugs. Our results on patient samples shows that it is effective in enhancing apoptotic cell death in primary AML cells even in AML sample with low sensitivity to these agents. For any anticancer therapy, it is important to use agents that target cancer cells and have minimal toxicity to untransformed cells. Notably, single drug treatment with either Resveratrol or HDACIs resulted in minimal toxicity, Resveratrol and HDACIs didn't show enhanced lethality in normal CD34+ cord blood cells, indicating the selectivity of this regimen. This Resveratrol and HDACIs combination induced lethality involves several mechanisms: inducing mitochondrial injury evident by marked increase of cytochrome *c* into the cytoplasm, induction of DNA damage, and caspase cascade activation. As previously described, these mechanisms have roles in establishing the anticancer activity of both Resveratrol and many HDACIs. Specifically, previous studies showed that extrinsic pathway is involved in HDACI induced lethality (144). In the light of the evidence that disrupting caspase8 activity cause significant protection of U937 cell against cell death, it is apparent that extrinsic pathway has a central role in Resveratrol+HDACIs combination induced apoptosis. Exposure of U937 cells to LBH or SAHA induced a clear upregulation of DR4 (41). However, co-administration of Resveratrol did not further enhance such events. The question how this combination treatment triggers activation of extrinsic apoptotic signaling pathway still remains unknown. In this context, it is known that HDACI-mediated upregulation of death receptors accounts for synergy between HDAC inhibitors and death receptor ligand (e.g., TNF α , TRAIL) (145) or other agents that can increase production of the secretive factors.(41) Therefore, one possibility arises that Resveratrol may also increase generation of one or more of these factors that binds to and activate death receptors, which are upregulated by HDACI, thereby trigger extrinsic pathway, and lead to enhanced lethality. This could also be explained by the fact that other death receptors could regulate the extrinsic pathway and also the cross talk between the intrinsic and the extrinsic pathways (146).

As was expected, Resveratrol administration promoted deactivation of NF- κ B through deacetylation. However, combination-induced cell death did not appear to be significantly dependent on SIRT1 promoted NF- κ B deacetylation as the down regulation of SIRT1 using shRNA increases NF- κ B acetylation but failed to protect U937 cell against HDACIs and/or

Resveratrol activity. This could be explained by the fact that SIRT1 is known to be involved in many pro-survival and metabolic homeostasis(147). To date; the precise mechanisms of SIRT1 action are still poorly understood. Also Resveratrol per se has anticancer effects that are not dependent on SIRT1 regulation, for example pro-oxidant effect and cell cycle arrest (105). The p21 short-lived anti-apoptotic and cell cycle protein is also important in the stress response (148). Previous studies showed that p21 can be induced by HDACIs and this induction is at least partly NF- κ B dependent and that downregulation of p21^{CIP1} promote HDACI-mediated lethality (25). Combination treatment of RESV and HDACIs results in significant block of HDACIs p21 upregulation (Data not shown). This blockade could be partially through deactivation of NF- κ B. Moreover, p21 downregulation could have an effect on the observed cell cycle disruption as discussed later. While Resveratrol has long being known to act as antioxidant, it can also increase ROS production (at least in tumor cells). For example, many studies showed that Resveratrol can enhance ROS production in different types of leukemia (149). The prooxidant action of Resveratrol may play a critical role in induction of apoptosis through ROS mediate DNA damages (113). On the other hand, ROS generation by DHACIs is one of the mechanisms by which these agents induce lethality (55). In our study, DHACIs generate moderate amount of ROS while significant increase in ROS generation was observed in Resveratrol or the combination treatment. Blocking ROS generation by the MnSOD2 antagonist TBAP co-incubation results in marked decrease in combination induced cell death. This finding implies the role of ROS generation in RESV+HDACIs induced lethality. Even though the difference in ROS generation between Resveratrol and the combination treatment was not very dramatic or sustained, ROS generation appear to play a significant role in promoting induced cell lethality through induction of caspase activation or by making U937 cells more sensitive to HDACIs lethality when co-administrated with Resveratrol.

Both HDACIs and Resveratrol promote perturbations in cell cycle regulations discussed above (67). Resveratrol was shown to cause alteration in many cell cycle-specific proteins that might affect cancer cell growth and proliferation. G1/S and G2/M checkpoints, are also important targets for Polyphenols (119). Previous studies had shown that Resveratrol affects cell cycle in different leukemic cell lines through S-phase arrest and inhibition of DNA synthesis (67, 125). Polyphenol treatments have various regulatory actions on cyclins, cyclins-dependent kinase (CDK), including p21 in leukemic cell lines (105, 126). In our study, minimal toxic doses

of SAHA didn't induce marked disruption of cell cycle however Resveratrol induces dramatic increase in S phase at 24 hrs, followed by markedly increased G2M population. Notably, these events were largely abrogated when HDACI was co-administrated, accompanied by a sharp increase in apoptosis. This interesting finding raises one of the possible mechanism by which Resveratrol enhances HDACIs lethality via trap cells in S phase which is sensitive to lethal effects of HDACI, increases S phase recruiting more cells into DNA damaging.

Recent studies also revealed that Resveratrol induced autophagic cell death (150) and unfolded protein response UPR (151) in leukemic cell lines. These finding could explain mechanisms by which Resveratrol mediates its chemotherapeutic effects. To this end, we also examined the possibilities that these mechanisms might also contribute to the interactions between Resveratrol and HDACIs. However, these phenomena were confirmed in U937 cells. However, there was no evidence for involvement of ER stress or autophagic cell death in RESV+HDACIs lethality (Data not shown).

In conclusion our results support the notion that combining the natural Polyphenol Resveratrol with the HDAC inhibitor Vorinostat and Panobinostat provide a potential strategy for AML treatment. This regimen was effective toward both AML cell lines as well as primary patient samples, while largely sparing normal hematopoietic cells (e.g., cord blood CD34+ cells). This combination regimen, exhibits synergistic interaction against human leukemia cells, most likely via multiple mechanisms, including induction of mitochondrial injury and DNA damage, activation of extrinsic caspase pathway, ROS generation, S phase-related sensitization, and NF- κ B inhibition. However, although SIRT1 is required for Resveratrol-mediated deacetylation of RelA/p65, our initial hypothesis for functional roles of SIRT1 activation in Resv-mediated enhancement of HDACI lethality remains uncertain. Since SIRT1 is a multifunctional protein, which mediates deacetylation of, in addition to RelA/p65, many other proteins, other approach(s), alternative to shRNA knockdown, may be required to address this issue. Nevertheless, since many HDACIs e.g. Vorinostat (Zolinza) are clinically approved for treatment of T-cell lymphoma, and Resveratrol has already been in use as a dietary supplement, our results provide clear evidence that this combination strategy has promising anti-leukemia activity, which warrants further attention in preclinical studies and clinical trials.

5. References

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