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OXIDATIVE STRESS BASED STRATEGIES FOR ENHANCING THE EFFICACY OF HISTONE DEACETYLASE INHIBITORS (HDACi)

Nilsa Rivera-Del Valle

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by

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OXIDATIVE STRESS BASED STRATEGIES FOR ENHANCING THE EFFICACY OF HISTONE DEACETYLASE INHIBITORS (HDACi)

A

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by

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Houston, Texas

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DEDICATION

I want to dedicate this work to all the cancer patients. Especially to a beautiful angel Morgan Anderson, who during a difficult time in my life, helped me to put things in perspective. Also my family and friends: Irma Rivera-Cosme, Carmen A. Castro-Maysonet, Migdalia Nieves-Cardona, Iris Rosado-Aponte and Alma Y. Guerrero-Miller Carmen, who are or had been great fighter's against cancer.

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OXIDATIVE STRESS BASED STRATEGIES FOR ENHANCING THE EFFICACY OF HISTONE DEACETYLASE INHIBITORS (HDACi)

Publication No. _____

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Histone deacetylase inhibitors (HDACi) are anti-cancer drugs that primarily act upon acetylation of histones, however they also increase levels of intracellular reactive oxygen species (ROS). We hypothesized that agents that cause oxidative stress might enhance the efficacy of HDACi. To test this hypothesis, we treated acute lymphocytic leukemia cells (ALL) with HDACi and adaphostin (ROS generating agent). The combination of two different HDACi (vorinostat or entinostat) with adaphostin synergistically induced apoptosis in ALL. This synergistic effect was blocked when cells were pre-treated with the caspase-9 inhibitor, LEHD. In addition, we showed that loss of the mitochondrial membrane potential is the earliest event observed starting at 12 h. Following this event, we observed increased levels of superoxide at 16 h, and ultimately caspase-3 activation. Pre-treatment with the antioxidant N-acetylcysteine (NAC) blocked ROS generation and reversed the loss of mitochondrial membrane potential for both combinations. Interestingly, DNA fragmentation and caspase-3 activity was only blocked by NAC in cells treated with vorinostat-adaphostin; but not with entinostat-adaphostin. These results suggest that different redox mechanisms are involved in the induction of ROS-mediated apoptosis. To further understand these events, we studied the role of the antioxidants glutathione (GSH) and thioredoxin (Trx). We found that the combination of entinostat-adaphostin induced acetylation of the

antioxidant thioredoxin (Trx) and decreased intracellular levels of GSH. However, no effect on Trx activity was observed in either combination. In addition, pre-treatment with GSH ethyl ester, a soluble form of GSH, did not block DNA fragmentation. Together these results suggested that GSH and Trx are not major players in the induction of oxidative stress. Array data examining the expression of genes involved in oxidative stress demonstrated a differential regulation between cells treated with vorinostat-adaphostin and entinostat-adaphostin. Some of the genes differentially expressed between the combinations include aldehyde oxidase 1, glutathione peroxidase-5, -6, peroxiredoxin 6 and myeloperoxidase. Taken together, these experimental results indicate that the synergistic activity of two different HDACi with adaphostin is mediated by distinct redox mechanisms in ALL cells. Understanding the mechanism involved in these combinations will advance scientific knowledge of how the action of HDACi could be augmented in leukemia models. Moreover, this information could be used for the development of effective clinical trials combining HDACi with other anticancer agents.

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CHAPTER 1: INTRODUCTION

1.1 Leukemia

Despite improvements in medicine, cancer remains one of the leading causes of death in the U.S. It is estimated that in 2012, about 1.6 million new cases of cancer will be diagnosed in the U.S. and one of four deaths will be due to cancer [3]. Among these new cases, approximately one third will be amongst the pediatric population. Within this population, leukemia is the cancer with the highest incidence and mortality among pediatric patients [3].

Leukemia by definition is a cancer of the blood and bone marrow cells [4]. This type of cancer is usually classified based on the cell type or by how rapidly the disease progress or develops. In the case of the cell type, these can be: lymphocytic leukemias, which are developed from the lymphoid precursors or myeloid leukemias, which are developed from the myeloid precursors (Figure 1). In terms of disease progression, these can be divided into acute versus chronic leukemias. The term acute refers to a rapid tumor cell proliferation and accumulation of immature or blast cells (lymphoblasts or myeloblasts). In the case of chronic leukemias, the predominant tumor cells are mature blood cells and are characterized by a slow growth rate. Based on this basic definition there are four major types of leukemias: acute lymphocytic leukemia (ALL), acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL) and chronic myeloid leukemia (CML) [4].

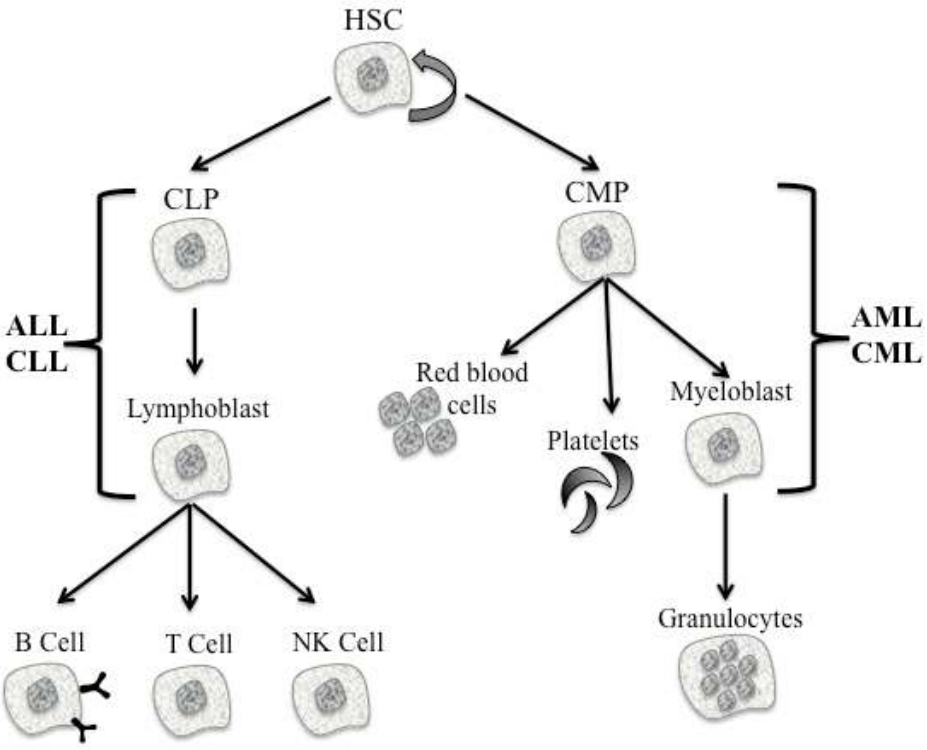


Figure 1. Origin of the major four classes of leukemias. Normal hematopoiesis (the formation of the blood) is a process where the hematopoietic stem cells (HSCs) differentiate into two common lineages: common lymphoid progenitors (CLP) and common myeloid progenitors (CMP). Acute Lymphoblastic Leukemia (ALL) and Chronic Lymphoblastic Leukemia (CLL) are derived from the CLP. In contrast, Acute Myeloid Leukemia (AML) and Chronic Myeloid Leukemia (CML) are derived from the CMP. Adapted from Irwin M.E. et al. Antioxidant and Redox Signaling 2012 Sep 28 [Epub ahead of print] [1].

The most common type of leukemias in children are acute leukemias, with ALL being the most common subtype. ALL comprises 80% of all childhood leukemias while AML accounts for approximately 18%. In terms of chronic leukemias, only about 2% of CML is diagnosed in children and CLL is rarely reported in pediatric patients [5]. The major cause of childhood leukemias has been linked to genetic factors and/or environmental factors. Some of the genetic factors include hyperdiploidy, hypodiploidy, chromosomal translocations and deletions [6]. In addition to these genetic abnormalities, several studies have found an association between genetic syndromes and the development of childhood leukemia. Some of these syndromes include: Down syndrome, neurofibromatosis and schwachman syndrome [7-9]. Environmental factors like in utero exposure to ionizing radiation; exposure to certain chemicals like benzene, hydrocarbons and pesticides has also been linked to the development of childhood leukemias [10, 11].

ALL is divided into different subtypes based upon morphological and cytogenetic features. In terms of morphological changes, ALL can be divided into three subtypes: ALL-L1, -L2 and -L3 [4]. In the L1 subtype the lymphoblast is relative small with a high nuclear to cytoplasmic ratio and small nucleoli. In L2 the lymphoblast is larger with low nuclear to cytoplasmic ratio and large nucleoli. In L3 the lymphoblast resemble a mature B cell with large vacuoles and nucleoli. Each of these subtypes are further divided by immunologic types: pre-B cells and pre-T cells [4].

Advances in the molecular genetics field have resulted in more accurate classification of ALL based on their genetic abnormalities. These cytogenetic classifications have helped to understand better the prognosis of ALL. Some of the cytogenetic features with good prognosis observed in ALL include: hyperdiploidy (more than 50 chromosomes) and translocations like t(12;21)(p21;q22). Otherwise

cytogenetic features with intermediate and poor prognosis include: hypodiploidy (less than 45 chromosomes), del(6q), del(9p), t(9;22)(q34;q11) and t(8;14)(q24;q32) [4].

During the past few decades, great advances in the treatment of pediatric leukemia has resulted in an 80% cure rate [12]. The treatment for pediatric leukemias varies according to age, disease stage, subtype and myeloid versus lymphoid origin. Standard treatment for leukemia includes chemotherapy, radiation, bone marrow transplantation, stem cell transplantation or a combination of these treatments [13]. Usually chemotherapy regimens are divided in three major stages or phases: induction, consolidation and maintenance phase [13]. During induction phase, the major goal is to kill the majority of the cancer cells. The duration of this phase is around three to eight weeks and during this time different chemotherapies are administered. The second phase is consolidation where the major goal is to eliminate any remaining cancer cells. The duration of this phase is around a few months. Finally after remission of the disease, the third phase of maintenance begins. This is a less intense phase and may last around one to two years. The major goal in this phase is to decrease the probability of relapse.

During all these phases different types of drugs are administrated. Since cancer cells are characterized by abnormal proliferation, the majority of the drugs used work by disrupting cell proliferation and progression of cell cycle. Some of the drugs most commonly used include anthracyclines, topoisomerase inhibitors and antimetabolites [13]. Anthracyclines like daunorubicin and doxorubicin are commonly used during induction phase and work by intercalating between base pair of the DNA inhibiting its synthesis. In addition some of these drugs can act as topoisomerase inhibitors, in this case the agent binds to the topoisomerase molecule blocking its ability to bind to the DNA, which ultimately results in DNA damage and cell death. In

the case of antimetabolites like methotrexate and thioguanine, these drugs work by preventing purines and pyrimidines to incorporate into DNA during S phase, preventing cell division and growth.

Even when cure rates are promising, 20% of pediatric patients with ALL are not curable due to specific molecular subtypes, which confer resistance to the current available chemotherapy. Furthermore, studies have demonstrated that survivors from pediatric leukemias tend to develop treatment related side effects later in life [14]. Some of the side effects observed include cardiotoxicity and the development of secondary cancers. In addition, even though ALL affects mostly pediatric patients, there is a group of adults that will develop ALL during the course of their life. The prognosis in these patients is not as promising as in children, resulting in a survival rate less than 40% [4]. For these groups of patients, the development of new selective drugs and strategies for the treatment of ALL is of great importance.

Conventional therapies, like the ones mentioned before, kill cancer cells by inhibiting growth. However, this inhibition is not specific for cancer cells; these drugs can also target normal cells that are actively dividing. Therefore, development of specific targeted therapies based on differences between cancer and normal cells is of utmost importance. The identification of specific subtypes and alterations in cancer cells have helped in the development of new targeted therapies. Some of the targeted therapies that have shown promise for the treatment of leukemias include: kinase inhibitors, histone deacetylase inhibitors, proteasome inhibitors and Bcl-2-targeted agents [15]. Interestingly, in addition to inhibiting their specific targets, some of these agents have been shown to alter the redox state of cancer cells [15, 16]. The following section will be dedicated to introduce the concept of oxidative stress and its implications for the treatment of cancer.

1.2 Oxidative stress

1.2.1 Redox homeostasis and oxidative stress

Redox homeostasis is defined as the balance between the production and elimination of oxygen-containing molecules named reactive oxygen species (ROS) [16]. ROS are highly reactive molecules that include radical species like superoxide and hydroxyl radical and non-radical molecules like hydrogen peroxide. The production of ROS within the cell is the result of different exogenous and endogenous stimuli. Exogenous sources of ROS include exposure to different chemicals and radiation. The major endogenous source of cellular ROS is the mitochondria, where superoxide is generated by the electron-transport chain and subsequent reactions result in the production of hydrogen peroxide and hydroxyl radical [16]. Other endogenous sources of ROS include the activation of oxidases including NADPH oxidase, xanthine oxidase and monoamine oxidase [16].

Proper redox homeostasis plays a critical role for the normal function of the cell. To maintain redox homeostasis, cells contain different antioxidant systems that help to eliminate excessive levels of ROS. Some of these antioxidants include enzymes like superoxide dismutase (SOD), which catalyze the conversion of superoxide to hydrogen peroxide and catalase. Subsequently catalase, another antioxidant, catalyzes the conversion of hydrogen peroxide to water and oxygen. Other non-enzymatic antioxidants like glutathione (GSH) and thioredoxin (Trx) play important roles in the elimination of ROS (Figure 2) [16].

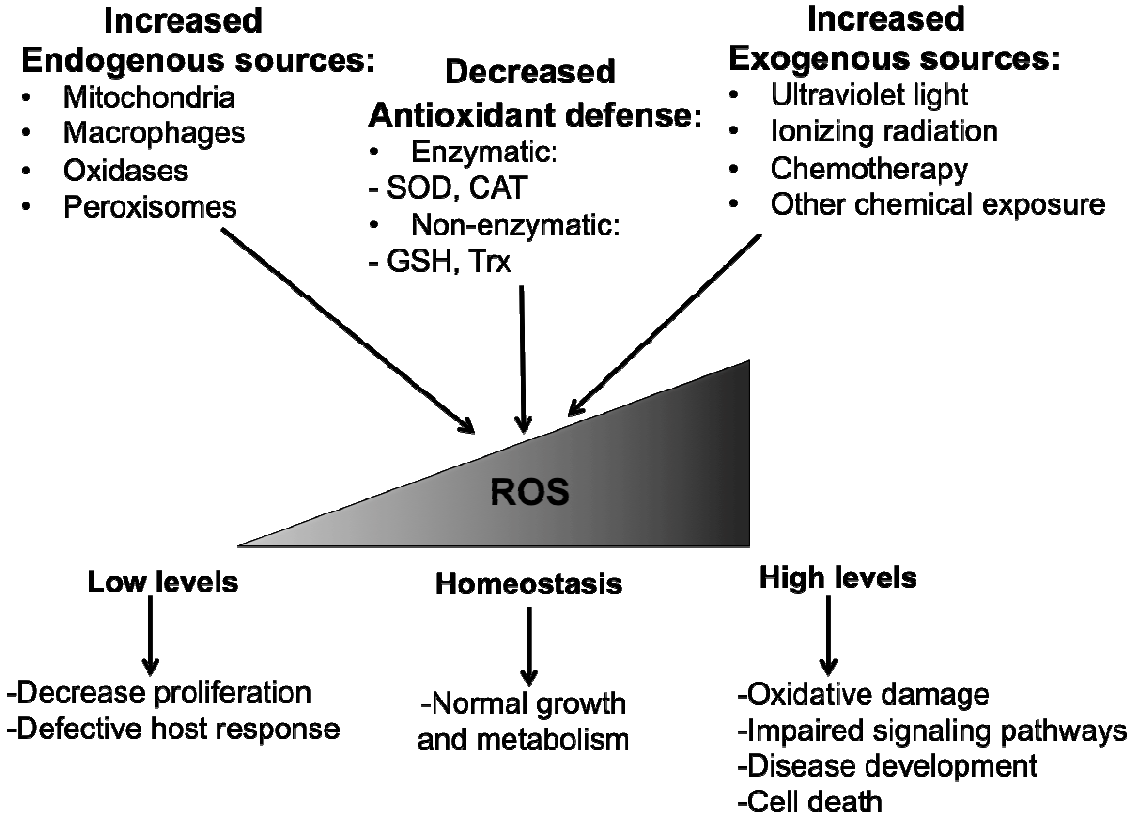


Figure 2. Sources of ROS and its cellular response. Production of ROS within the cell can be the result of endogenous or exogenous sources. To maintain homeostasis in the levels of ROS, cells contain antioxidant defense mechanisms. An increase in antioxidants like superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and thioredoxin (Trx) help the cells to maintain homeostasis. When levels of ROS decrease or increase, different physiological functions can be affected.

Glutathione (GSH)

GSH is a tripeptide consisting of glutamate, cysteine and glycine. These amino acids are assembled into active GSH through the enzymatic action of γ-glutamylcysteine synthetase and GSH synthetase [17]. This molecule is the most abundant thiol containing peptide within the cells. Its major function is to work as a reducing agent and ROS scavenger. A reactive sulfhydryl group drives its antioxidant function. In addition to working as an antioxidant, GSH plays different roles within the cell including metabolism of xenobiotics, cell-cycle regulation, control of mitochondrial mediated cell death, modulation of immune response and regulation of protein function and gene expression via thiol-disulfide exchange reactions [17]. Due to its important role in cell physiology, alterations in GSH levels have been associated with the development of different diseases, including neurodegenerative disorders, diabetes, cardiovascular diseases and cancer [18, 19].

GSH, serving as an electron donor, can reduce disulfide bonds-containing proteins to cysteines. As a ROS scavenger, GSH works by converting reactive species such as hydrogen peroxide to less reactive molecules. In this process, glutathione peroxidase (GPX) catalyzes the conversion of GSH to the oxidized glutathione disulfide (GSSG). Subsequently, GSSG can be reduced back to GSH by glutathione reductase in a reaction that requires NADPH as an electron donor (Figure 3) [17]. The ratio of GSH to GSSG within the cell is regularly used as a measurement of cellular toxicity [20]. In addition to functioning as an antioxidant, GSH also plays important roles in the generation of other antioxidants like vitamin C and E [17].

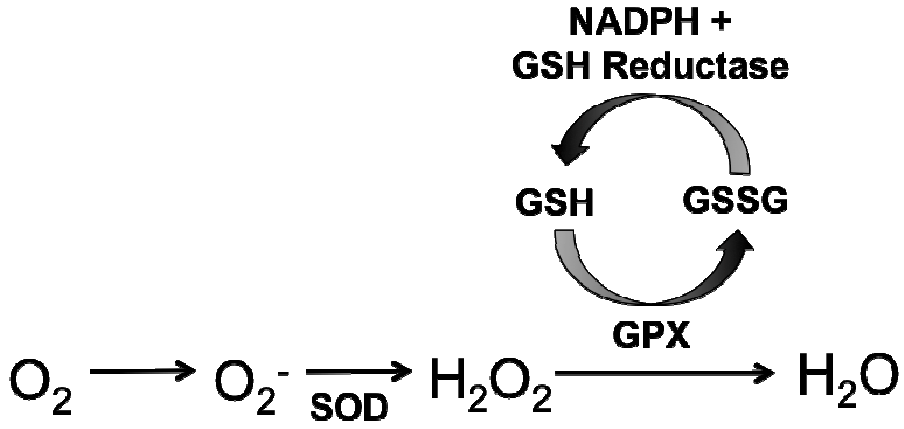


Figure 3. GSH antioxidant reaction. Superoxide (O_2-) is converted to hydrogen peroxide (H_2O_2) by a reaction catalyzed by superoxide dismutase (SOD). Using reduced glutathione (GSH) as a substrate, glutathione peroxidase (GPX) converts H_2O_2 to water (H_2O). During this reaction GSH is oxidized to glutathione disulfide (GSSG). GSSG can be reduced back to GSH by glutathione reductase using NADPH as a substrate.

Thioredoxin (Trx)

Trx is another important thiol containing antioxidant, which plays a role in maintaining redox homeostasis within the cell. Human Trx was originally cloned as an adult T cell leukemia (ATL)-derived factor; produced by a HTLV-1 transformed T cell line ATL-2 cells [21]. Trx is a 12 KDa selenoprotein containing two redox-active cysteines and a conserved active site. The Trx system is primarily composed of two isoforms of Trx, which include cytosolic Trx1 and mitochondrial Trx2. Two other major members of this family include Trx reductase-1 and -2 (TrxR-1 & TrxR-2), which are localized in the cytosol or the mitochondria respectively [22]. To maintain homeostasis Trx-binding protein 2 (TBP-2), also known as Trx-interacting protein (TXNIP), works as a negative regulator of Trx. This molecule binds directly to Trx, resulting in decreased antioxidant capacity [23]. Similar to GSH, Trx aids in the removal of hydrogen peroxide and plays an important role in reducing disulfide bond-containing proteins. In this process, peroxiredoxin catalyzes the conversion of reduced Trx (Trx-S_2) to its oxidized form (Trx-$(SH)_2$). Then Trx-$(SH)_2$ can be reduced back to Trx-S_2 by thioredoxin reductase (TrxR) using NADPH as an electron donor [22] (Figure 4A).

In addition to working as an antioxidant, Trx has pleiotropic functions within the cell. Trx has been known to act as a growth factor, activator of transcription factors and apoptotic controller [22]. This pleiotropic function is driven by its interaction with other proteins like the transcription factor NF-κB and the apoptosis signal-regulating kinase 1 (ASK-1) (Figure 4B). In the case of NF-κB, reduced Trx can bind to NF-κB, helping in its translocation to the nucleus and binding to DNA, which results in gene expression (Figure 4C) [24, 25]. This binding to the DNA is regulated by the reduction of a disulfide bond in cysteine 62 of the p50 subunit of NF-κB [26]. As an apoptotic

controller, reduced Trx can bind to ASK-1, which is required for TNF-α-induced apoptosis [27, 28]. The binding to ASK-1 results in inhibition of ASK-1 dependent apoptosis (Figure 4B). In the presence of TNF-α or generation of ROS, Trx is oxidized resulting in dissociation of ASK-1, subsequently leading to its activation and apoptosis.

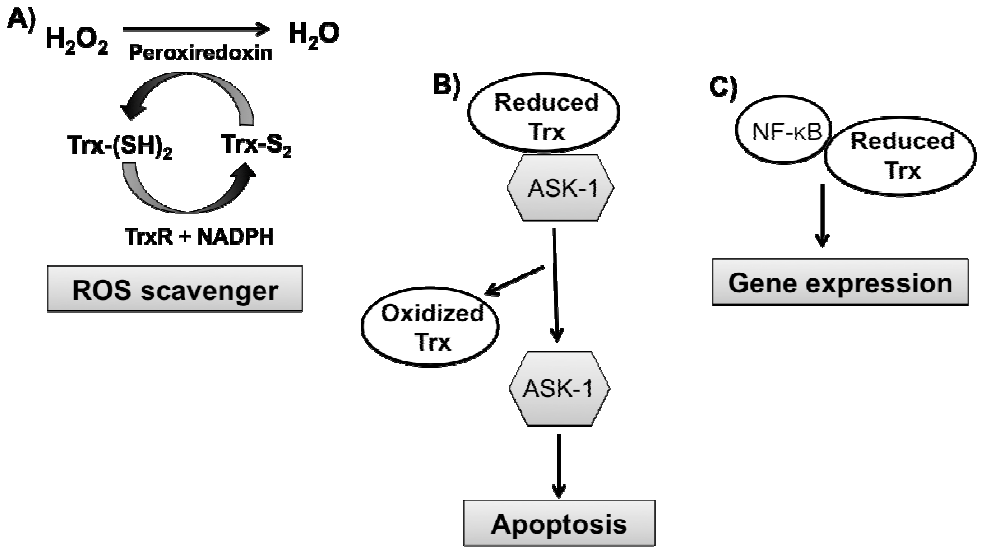


Figure 4. Pleiotropic effect of Trx. A) Trx works as a ROS scavenger by removing hydrogen peroxide (H_2O_2). This reaction is catalyzed by peroxiredoxin. B) Trx also regulates apoptosis by binding to apoptosis signaling-regulating kinase-1 (ASK-1). C) Reduced Trx can bind directly to NFκB affecting gene expression.

The balanced production of ROS and antioxidants is important to maintain redox homeostasis and for the proper function of the cell. An imbalance between antioxidants and oxidants results in oxidative stress, which can affect cell survival in different forms. Under oxidative stress, elevated levels of ROS can result in damage of lipid membranes, proteins and DNA [16]. Alterations in cellular metabolism and antioxidants levels have been associated with different human diseases including neurodegenerative diseases, cardiovascular disorders and cancer [16].

In the next section we will discuss the different redox alterations that can result in the development of cancer, focusing primarily on ALL.

1.2.2 Redox alterations in leukemia and its implication in cancer therapy

Several studies have demonstrated that cancer cells, including the cells of hematological malignancies, have higher levels of ROS than normal cells [29, 30]. These elevated levels of ROS have been associated with tumor initiation, promotion and maintenance of the cancer cells [16]. The exact mechanisms responsible for the redox alterations observed in leukemic cells are not clear. Several studies suggest that elevated levels of ROS in leukemia can be the result of oncogenic activation. The BCR/ABL oncogene, which is expressed in CML and a subtype of ALL, has been demonstrated to alter redox homeostasis by increasing the levels of peroxide and superoxide [31, 32]. In addition to BCR/ABL, other alterations like overexpression of the antiapoptotic protein BCL-2 has been associated with redox control in leukemia. BCL-2 can function as an oncogenic signal affecting the production of GSH resulting in redox alteration in ALL cells [33, 34]. In this case, inhibition of BCL-2 with the agent ABT-737 in ALL cells results in GSH depletion and increase oxidative stress [34].

Another explanation for the elevated levels of ROS observed in leukemias is the hyperactivation of endogenous sources of ROS such as the mitochondrial electron chain. Mutations in mitochondrial DNA have been observed in different leukemias [35, 36]. These mutations can alter the electron transport chain resulting in oxidative stress. Furthermore, ROS production by complex I of the mitochondrial electron chain was reported as a survival factor in ALL patient samples [37].

As observed in other cancer cells, leukemia cells appear to have altered expression or dysregulation of antioxidant proteins. This dysregulation can result in elevated expression of antioxidants, which allows leukemic cells to readily cope with the increased ROS production. Antioxidants that have been reported to be up-regulated in ALL include GSH and Trx. Different studies have compared healthy donor samples with leukemia patient samples. The results demonstrated higher GSH levels in CLL, AML and ALL patients compared to healthy individuals [38, 39]. Several studies have also associated elevated levels of GSH with resistance to chemotherapy in ALL cells [40, 41]. Furthermore, there is a correlation between elevated GSH and an increased risk of relapse in pediatric patients with ALL [42].

Trx dysregulation has also been demonstrated to alter the redox status in leukemias. Trx is upregulated in leukemias resulting in sustained growth and decreased apoptosis [43]. Studies in acute leukemia patient samples have found a correlation between elevated Trx mRNA expression with relapse and poor prognosis, suggesting Trx is a negative prognostic indicator [44]. The activation of Trx pathways in acute leukemia seems to be different between leukemia types. In AML patient samples, TBP-2 is overexpressed resulting in decreased Trx activity and elevated ROS [45]. In contrast, TBP-2 is lost in ALL and consequent upregulation of Trx is observed. In this study re-expression of TBP-2 resulted in growth inhibition [46].

On the other hand, dysregulations resulting in decreased levels of antioxidants can lead to elevation of ROS levels. Consequently this elevation in ROS can result in proliferation and genomic instability. SOD, is an antioxidant, which catalyzes the conversion of superoxide to hydrogen peroxide and catalase. Studies have reported decreased levels of SOD in lymphocytes from ALL patients when compared to healthy donors samples [47, 48]. Another example of decreased levels of antioxidants in

leukemia includes catalase. Catalase is an enzyme involve in the elimination of hydrogen peroxide. ALL cells have lower levels of catalase activity compared to normal cells. These results were associated with an increase in hydrogen peroxide and genomic instability [48].

These redox alterations in cancer cells have been exploited for the identification and development of new specific and selective anticancer treatments. It has been hypothesized that if a chemotherapeutic agent has the ability to push ROS levels in malignant cells past a particular threshold, cell death will occur [29]. In contrast, this increase in ROS will not cause major damage to normal cells due to their low basal ROS levels. Some of the chemotherapeutic agents that have been shown to induce oxidative stress mediated apoptosis in leukemia cells include: proteasome inhibitors, Bcl-2-targeted agents, kinase inhibitors and HDACi [49, 50]. The following section focuses on HDACi as a targeted agent inducing oxidative stress mediated apoptosis.

1.3 Histone deacetylase inhibitors (HDACi)

1.3.1 Rationale for the development of HDACi

Cancer has traditionally been defined as a genetic disease where genetic alterations have been considered as a major cause of carcinogenesis [51]. Carcinogenesis or development of cancer consists of three major steps: initiation, promotion and progression. During the initiation step, genetic alteration or mutations result in the formation of cancer cells. In the promotion step, the cancer cells are clonally expanded and finally in the progression step additional genetic mutations can be acquired. Mutations in oncogenes and tumor suppressor genes may result in changes in gene expression, which has been attributed to the development of cancer.

In the past few years, completion of the human genome sequence has led to the study of epigenetics. Epigenetics is defined as a heritable change in gene expression without alterations in the underlying DNA sequence [52]. Several studies have demonstrated that as genetic alterations, epigenetic changes in gene expression can be involved in the process of carcinogenesis [53]. While many genetic defects, such as gene deletions, cannot be reversed, many epigenetic abnormalities in cells can be reversed, making epigenetic changes attractive targets for the development of new therapies. Two of the most common epigenetic alterations observed in cancer are DNA methylation and post-translational modification of histones.

DNA methylation is the most studied epigenetic alteration in cancer. The process of DNA methylation involves DNA methyltransferases (DNMTs), which transfer methyl groups from S-adenosylmethionine to cytosines that are part of CpG dense regions or better known as CpG islands [54]. The methylation of these regions is associated with transcriptional repression. Studies have demonstrated that cancer cells exhibit different patterns of DNA methylation in genes involved in important cells

functions like cell cycle regulation, DNA repair, and tumor suppression and/or histone modification compared to normal cells [55-57].

The second most studied epigenetic alteration includes the post-translational modifications of histones. Histones are proteins found predominantly within the cell nucleus and the major role is to package the DNA to form the nucleosome, which is the basic building block of the chromatin. Histone modifications include: methylation, acetylation, phosphorylation, ubiquitination and ADP-ribosylation [58]. These modifications give the nucleosome a structure where different processes like transcription, DNA replication, and DNA repair take place.

Histone acetylation has been identified as a target for the treatment of cancer. The acetylation of histones is a dynamic process, which include two different enzymes: the histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Figure 5). The HATs are divided into five families, which include the GNAT superfamily, the Myst family, p300/CBP, TAFII 250 and nuclear receptors [59]. These enzymes use acetyl-CoA to add acetyl groups to lysine residues on histone tails. The amine groups present in lysine confer a positive charge to histone tails [60]. This positive charge interacts with the negative charge found on the phosphate group of the DNA chain. Histone acetylation changes the amine groups to amides, which neutralizes the positive charge on the histone tail resulting in decreased ability of histones to bind the DNA chain [60]. The decrease interaction of histone with the DNA chain confers a more relaxed chromatin structure allowing different transcription factors and molecular complexes to interact with the chromatin resulting mainly in gene transcription [61].

In contrast, the HDACs, which are divided into four classes (Figure 6), remove acetyl groups. The removal of acetyl groups increases the positive charge of histone which then can interact with the DNA chain resulting in a more compacted chromatin,

which is associated with transcriptional repression [60]. In addition to histones, HDACs can acetylate lysine residues on non-histone proteins like transcription factors, chaperone proteins and other proteins involved in cell signaling [62]. This acetylation in non-histone proteins can interfere with binding, activity or stability of the protein.

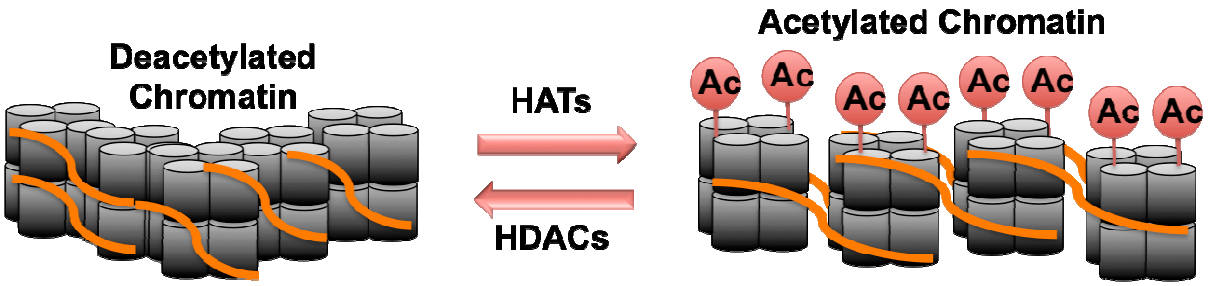


Figure 5. Chromatin acetylation. Acetylation of histone tails is driven by the homeostasis between histone acetyltransferases (HATs) and histone deacetylases (HDACs) enzymes. HATs add acetyl groups to the histone tails allowing a more relax chromatin structure. HDACs remove the acetyl groups resulting in a more compacted chromatin.

Different studies have demonstrated the association of abnormal expression of HDAC with cancer [63]. Histone hypoacetylation has been associated with the silencing of important genes like tumor suppressor genes and genes involved in cell differentiation and apoptosis [64]. Also, loss of acetylation of lysine 16 in histone H4 has been associated with cancer [65]. Furthermore, studies suggest a possible relation between histone modification patterns and poor outcome in different types of cancer [56, 66]. As results of all these observations, new therapies that target epigenetic changes in cancer have been developed. One of these emerging new therapies includes the histone deacetylase inhibitors (HDACi).

HDACi are a group of compounds that block the activity of HDACs resulting in hyperacetylation of histones or non-histone proteins. This group of inhibitors includes

natural and synthetic derivates, which are grouped on the basis of chemical structure and their ability to inhibit the activity of specific HDACs (Figure 6).

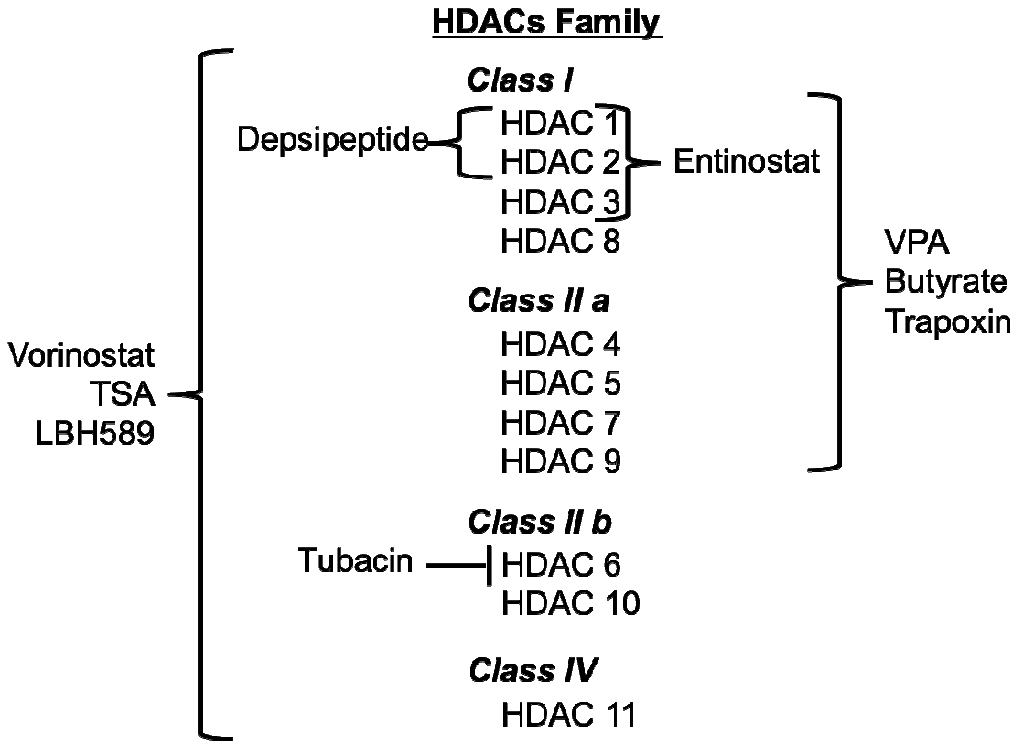


Figure 6. HDAC enzyme family. HDAC are divided in four classes based on their catalytic domain and cellular localization Class I, II a, II b and IV. Different HDAC inhibitors have been developed to target specific HDAC. Pan-HDAC inhibitors include: vorinostat, Trichostatin A (TSA) and panobinostat (LBH589). Inhibitors selective for class I HDAC include: entinostat and depsipeptide. Class I and II a inhibitors include: valproic acid (VPA), butyrate and trapoxin. More specific inhibitors like tubacin inhibit specifically HDAC 6, a member of the Class II b.

These compounds were discovered through different observations where treatment with dimethyl sulfoxide (DMSO) and the antibiotic trichostatin A (TSA) induced cell differentiation and cell cycle arrest in murine erythroleukemia cells [67, 68]. More detailed observations demonstrated that TSA targets HDACs resulting in their inhibition and consequently histone hyperacetylation [68]. Based on these

observations different efforts have been directed to develop agents with similar activity observed by DMSO and TSA treatments. Presently, there are approximately six classes of HDACi, including short-chain fatty acids, hydroxamic acids, and benzamide [69]. The efficacy of HDACi in inhibiting the enzymatic activity of HDACs varies between classes (Figure 6).

Vorinostat, or originally named by its chemical structure as suberoylanilide hydroxamic acid (SAHA; Figure 7) is the first HDACi approved by the U.S. Food and Drug Administration for treatment of cutaneous T-cell lymphoma.

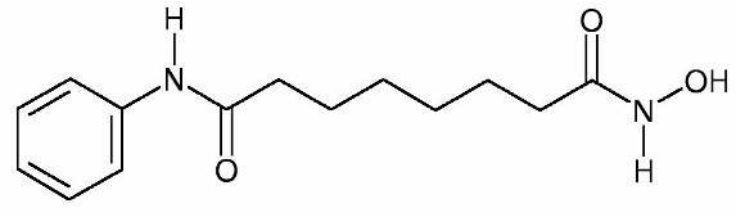


Figure 7. Chemical structure of vorinostat. Vorinostat or SAHS is a hydroxamic acid, which is a pan-HDAC inhibitors**.** *www.lclabs.com*

This agent was first identified as an HDACi by Richon et al., where they reported that vorinostat inhibited the activity of HDACs 1 and 3 in cancer cells [70]. Subsequent studies demonstrated that vorinostat works as a pan-HDAC inhibitor blocking the enzymatic activity of class I, II and IV HDAC [71]. In vitro studies have demonstrated that vorinostat has different biological effects including cell cycle arrest, cell differentiation, induction of apoptosis, angiogenesis inhibition and modulation of immune response [71]. In addition to increasing histone acetylation, vorinostat has the ability to induce non-histone proteins. Increased acetylation of different transcription factors like p53, E2F and HIF-1α and cytoplasmic proteins like tubulin and HSP90 have been associated with the mechanism by which this agent induces cell cycle arrest and cell death [71]. The anticancer effect of vorinostat has been also tested in

in vivo models. In a xenograft model of prostate cancer, administration of 50 mg/kg vorinostat resulted in significant suppression of tumor growth when compared to mice receiving vehicle alone [72]. In an *in vivo* model of ALL, vorinostat was administrated at different doses including 50, 100 and 150 mg/kg resulting in growth suppression of the xenograft tumors [73].

Entinostat is a novel HDACi, which is currently in clinical trials for the treatment of different cancers. Entinostat, previously known as MS-275, is a benzamide with selective inhibition of class I HDAC, specifically HDAC 1, 2 and 3.

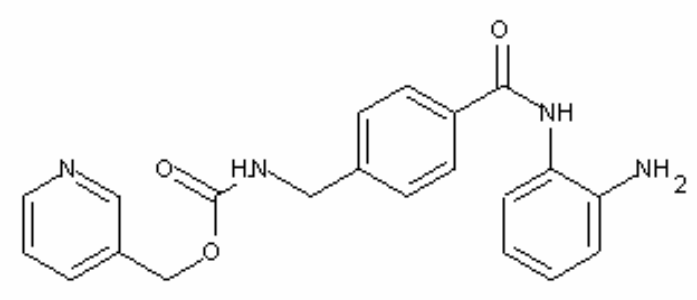


Figure 8. Chemical structure of entinostat. Entinostat or MS-275 is a benzamide which inhibits class-I HDACs**.** *www.exchemistry.com*

Different studies have demonstrated that this agent induces cell cycle arrest, cell differentiation and apoptosis in cancer cells [74]. In vitro studies using leukemia cell lines and patient samples demonstrated that lower doses of entinostat induced p21-dependent growth arrest. In contrast, treatment with higher concentrations of this agent induced apoptosis in these samples [75]. This agent also has the ability to target leukemic oncogenes like FLT3-ITD. FLT3-ITD is an oncogene associated with acute leukemias. In a study using FLT3 positive leukemia cells, entinostat induced acetylation of HSP90 and ubiquitination of FLT3 resulting in degradation of FLT3 and cell death [76]. Another mechanism by which this agent can induce growth arrest is

through the Akt pathway. Entinostat blocked the Akt signaling in AML cells resulting in growth arrest and cell differentiation [77]. In vivo studies using different cancer model, including sarcoma and neuroblastoma, demonstrated that administration of entinostat inhibited the growth of established tumors [74].

In general, the rationale behind the development of HDACi is that these drugs will increase acetylation of histones, which may lead to the reactivation of important genes involved in cell proliferation, cell differentiation, modulation of the immune response and induction of cell death. Interestingly, several studies have demonstrated that HDACi also induce oxidative stress as one of its mechanism of action. The following two sections will discuss the mechanism by which HDACi induce apoptosis and oxidative stress.

1.3.2 Induction of apoptosis by HDACi

In general, HDACi have been demonstrated to induce apoptosis in different types of cancer cells, including leukemic lymphocytes and myelocytes [78]. Apoptosis is defined as programmed cell death, which is physiological necessary to eliminate unwanted cells within an organism [79]. This type of cell death is characterized by changes in the cells that include: cell shrinkage, disruption of the mitochondria, DNA fragmentation and the appearance of apoptotic bodies. These apoptotic bodies are recognized by phagocytes, which finally engulf and eliminate the dead cell.

The process that directs all these changes within the cells is very complex and organized. There are two major pathways by which the cells undergo apoptosis: the extrinsic pathway (death-receptor pathway) and the intrinsic pathway (mitochondrial pathway) [79]. Both of these pathways are driven by different proteases better known as caspases (cysteine-directed aspartate specific proteases). These enzymes

recognize and cleave their substrates at tetra-peptide sites that have specific sequences. Caspases are divided into initiators (activator) caspases, effector (executioner caspases) depending on their function [79].

The extrinsic pathway is mainly driven by the initiator caspase-8. This pathway is initially activated by the binding of FasL, TRAIL or TNF-α to death receptors localized at the cell membrane. This binding results in the oligomerization of the death receptor and the internal recruitment of the adaptor protein FADD with the caspase-8. The complex of FADD and caspase-8 form the death-inducing signaling complex (DISC), which initiates the apoptotic process through the cleavage of caspase-8 or -10. The activation of caspase-8 or -10 induces the activation of the effector caspases-3 or -7, which ultimately results in all the biochemical and morphological changes associated with apoptosis [79].

The intrinsic pathway is regulated by pro-apoptotic and anti-apoptotic proteins. Pro-apoptotic proteins like Bax can activate the intrinsic pathway by binding to the mitochondrial membrane. This binding results in mitochondrial membrane perturbation and the release of cytochrome c, which then associates with procaspase-9 and the adaptor protein Apaf-1 to form the apoptosome. The apoptosome, activates the initiator caspase-9 which ultimately cleaves and activates the executors, caspase-3 or -7 resulting in cell death. The intrinsic pathway can also be activated by the extrinsic pathway. In this case the pro-apoptotic protein Bid serves like a link between the two pathways. Bid is cleaved and activated by caspase-8 resulting in the fragment tBid, which then binds to the mitochondrial membrane resulting in the activation of the intrinsic pathway. Depending on the target cell and the specific HDACi, different

studies have demonstrated that HDACi can induce apoptosis in cancer cells by the two-apoptotic pathways, the extrinsic and the intrinsic pathway.

In terms of the extrinsic pathway, in vitro studies using AML cell lines and patient blast samples, demonstrated that vorinostat and entinostat induce the gene expression of TRAIL by directly activating the TNFSF10 promoter, which consequently activates the extrinsic apoptotic pathway by binding to death receptors [80]. However this effect was not observed in normal CD34+ cells, which were resistant to HDACi induced-apoptosis [80]. Furthermore, in vivo studies validated the previous observations, demonstrating that suppressing TRAIL and Fas by siRNA resulted in significant reduction in apoptosis after treatment with HDACi [81]. In addition to these observations, Guo et al. demonstrated that treatment of ALL cells with the HDACi LAQ824 increases the mRNA and protein expression of the death receptors DR5 and DR4 [82]. In contrast, studies using lymphoma cells found that knockout of TRAIL or inhibition of caspase-8 activation have no effect on the apoptotic induction by LAQ824 and LBH-589 [83]. These last observations suggest that the activation of the extrinsic pathway by HDACi may be cell-type dependent. Other mechanisms by which HDACi can activate the extrinsic pathway can be through the recruitment of FADD and activation of caspase-8. Treatment of TRAIL resistant CLL cells with TSA and romidepsin resulted in sensitization of these cells to TRAIL induced apoptosis through the recruitment of FADD and caspase-8 into the DISC [84].

In addition to the extrinsic apoptotic pathway, HDACi have demonstrated to induce apoptosis through the intrinsic apoptotic pathway. These drugs can transcriptionally activate BH3-only proteins including Bid, Bad and Bim, which are important proteins in the activation of the intrinsic pathway [85-87].

Treatment of leukemia patient samples with vorinostat induced the cleavage of caspase-8, -9 and -3. However, the overexpression of Bcl-2 in these cells resulted in complete inhibition of vorinostat-induced apoptosis [88]. Further studies using an in vivo model of Burkitt's lymphoma, demonstrated that overexpression of Bcl-2 resulted in resistance to HDACi, again validating the importance of the prosurvival Bcl-2 protein [89]. Studies have demonstrated that HDACi can also induce the cleavage of Bid resulting in disruption of the mitochondrial membrane, release of ROS and cytochrome c and ultimately apoptosis in leukemia cells [87].

In addition to inducing apoptosis by the up- or down-regulation of apoptotic related proteins, several studies have demonstrated that HDACi induces oxidative stress in different types of cancer cells [87, 90, 91]. However the mechanisms by which HDACi induce oxidative stress is not well understood. These studies suggest a possible role of redox regulation in the efficacy of HDACi alone and in combination with other anticancer agents. As presented in previous sections, redox alterations in cancer cells can be used as a strategy to treat cancer. Understanding how agents like HDACi can alter the redox status in cancer cells is of critical importance for the development of more effective agents, in this case HDACi. Furthermore understanding of these mechanisms can help to anticipate possible drug resistance and better design of clinical trials that include combinations of HDACi with other anticancer agents. The following section will discuss the literature that support the mechanisms by which different HDACi induce oxidative stress.

1.3.3 Induction of oxidative stress by HDACi

The mechanisms by which HDACi induce oxidative stress is not well understood. Two possible mechanisms have been reported. The first one reported vorinostat to induce apoptosis by the expression of Bid, a pro-apoptotic protein, which resulted in disruption of the mitochondria and consequent production of ROS (Figure 4A) [69]. The second mechanism involved the antioxidant thioredoxin (Trx). Studies have shown vorinostat to inhibit Trx as a mechanism by which oxidative stress may occur [92]. An explanation for this Trx inhibition is that vorinostat increased the expression of thioredoxin-binding protein (TBP-2), which binds and inactivates Trx (Figure 4B). Furthermore, additional studies showed that non-transformed cells displayed higher levels of Trx compared to transformed cells. Non-transformed cells with higher levels of Trx were more resistant against HDACi. Otherwise, transformed cells with lower levels of Trx were more sensitive to vorinostat and entinostat [93].

Interestingly, post-translational modifications of Trx, like glutathionylation [94], have been shown to affect its activity. A recent study reports Trx to be acetylated by vorinostat [62], however it is unknown if Trx acetylation affects its activity. Trx acetylation and consequent Trx inhibition may work as a novel mechanism of how HDACi induce oxidative stress in cancer cells and will be tested in this dissertation.

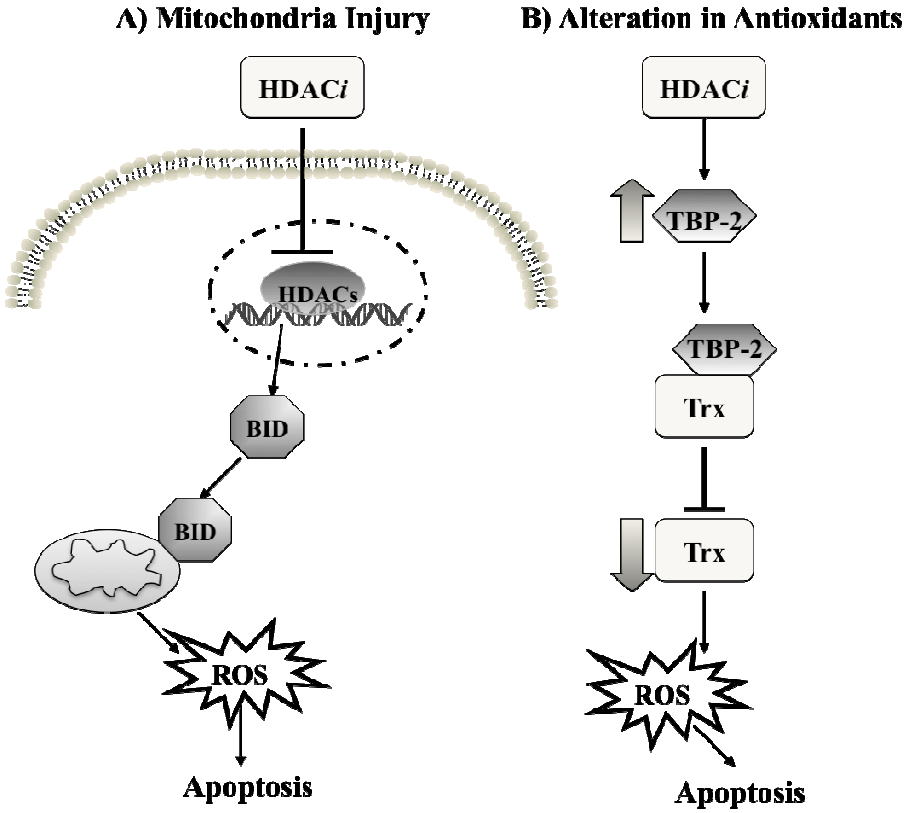


Figure 9. Proposed mechanism of ROS induction by HDACi. A) HDACi can induce the expression of BID, which associates with the mitochondrial membrane resulting in release of ROS. B) HDACi can induce the expression of TBP-2, which binds to Trx inhibiting its activity resulting in ROS accumulation [2].

1.3.4 HDACi in the clinic

As presented in previous sections, HDACi have gained widespread attention due to its anticancer activity in preclinical studies. In addition, multiple clinical trials using HDACi as a single agent and in combination with other chemotherapies have yielded promising results. This section will discuss some of the clinical trials using HDACi.

Vorinostat was the first HDACi showing positive results in clinical trials. This agent was approved for the treatment of cutaneous T-cell lymphoma. During phase I and II trials, vorinostat was well tolerated and 30% of the patients enrolled in the trial responded positively to the treatment [95]. These trials resulted in the approval of vorinostat for the treatment of this type of cancer. Recently, clinical trials using vorinostat have evaluated its efficacy in different types of leukemias. A phase I trial of vorinostat in AML patients demonstrated effective inhibition of HDAC activity in blood and bone marrow samples [96]. A second phase I trial evaluated the maximum tolerated dose (MTD) and the side effects of vorinostat in pediatric leukemia patients [97]. The MTD reported in these patients was 230 mg/m^2/d administrated orally and the side effects included nausea, loss of appetite and fatigue. Overall, this trial concluded that the pharmacokinetics of vorinostat in children was similar to that observed in adult patients [97]. Gojo et al. reported a phase I trial using entinostat in adults with refractory and relapsed acute leukemias. In this study, entinostat effectively inhibited HDAC activity, induced p21 expression, histone acetylation and caspase-3 activation in bone marrow cells [98]. In summary, these studies demonstrate HDACi to be promising for the treatment of hematological malignancies.

In addition to hematological malignancies, several clinical trials have been conducted to test these agents in patients with glioblastoma, ovarian, breast, colorectal and lung cancer [99-101]. In contrast to patients with hematological malignancies, these trials resulted in moderate or no effect. Possible explanation for the efficacy differences between liquid and solid tumors include the inability to achive appropriate doses of HDACi and consistent acetylation of target proteins. In clinical trials vorinostat's half-life is relatively short, 91.6 to 127 minutes administrated orally and 34.7 to 42.4 minutes administrated intravenously [102, 103]. These data suggest that continuous administration of this agent may be necessary to achieve clinical response.

While vorinostat has been approved as a single agent for the treatment of cancer, several preclinical and clinical studies have demonstrated that HDACi work better in combination with other anticancer agents [104]. HDACi have been combined with conventional cytotoxic therapies like DNA-damaging and targeted therapies like proteasome inhibitors and hypomethylating agents [105-107]. These combinations produced enhanced apoptosis in different malignant cell lines. Several clinical trials are presently being conducted using HDACi in combination with other chemotherapies; however, the mechanism of action of these combinatorial treatments is not well understood.

As mentioned before, HDACi have been shown to induce oxidative stress in different cancer cells. Knowing that cancer cells have higher levels of ROS compared to normal cells, this difference can be used as a therapeutic strategy to treat cancer. Combining HDACi with agents that cause further oxidative stress might enhance the efficacy of HDACi for the treatment of cancer.

Different studies combining HDACi with other chemotherapies have shown to induce oxidative stress, however the mechanism by which these combination induce oxidative stress is not very well understood. Rosato RR, et al. reported that the HDACi LAQ-824 in combination with fludarabine synergistically increased ROS levels in ALL cells. This increase in ROS resulted in DNA damage [108]. Oxidative stress is also a marker for the synergy observed between entinostat and the chemotherapeutic agent 5-azacytidine in AML and ALL cells [109]. PEITC is another compound that induce oxidative stress and has been combined with HDACi. In this study, depletion of GSH by PEITC increases sensitivity to vorinostat in leukemia cells [110]. Furthermore, the combination of HDACi with PEITC also induced ROS accumulation and apoptosis in a vorinostat-resistant cell line via activation of the NADPH oxidase. Induction of ROS by this combination allows for the translocation of the transcription factor Nrf2 to the nucleus, stimulating transcription of genes involved in the GSH system [110].

1.4 Adaphostin as a ROS generating agent

Adaphostin has been identified as a potential anticancer agent to treat acute leukemias such as ALL and AML. This molecule is a second-generation tyrphostin kinase inhibitor, which mimics tyrosine binding to kinases [111]. While this agent was developed as a BCR/ABL kinase inhibitor, subsequent studies have demonstrated that adaphostin's antileukemic activity is not selective for the presence of Bcr/Abl kinase [112]. Importantly, adaphostin demonstrates selectivity for leukemia cells as compared to normal lymphocytes [113]. Subsequent studies have been devoted to understanding the mechanism of action of this drug.

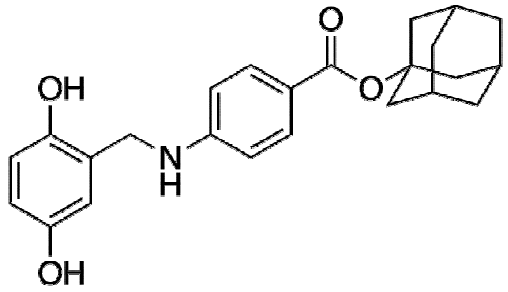


Figure 10. Chemical structure of adaphostin. Adaphostin is a second-generation tyrphostin kinase inhibitor. *www.trc-canada.com*

Mechanistic studies have demonstrated that adaphostin increases levels of intracellular ROS by the depletion of GSH, resulting in DNA damage and induction of apoptosis in leukemia cells [113]. More detailed investigations have explained the induction of oxidative stress by adaphostin. Le, S.B. et al reported that the increase in ROS in cells treated with adaphostin is the result of an accumulation in the mitochondria, where adaphostin binds to complex III, inhibiting electron transport [114]. Moreover, transcriptional and proteomic analyses of adaphostin-treated cells demonstrated an upregulation of oxidative stress-related genes [115, 116].

Hose, C. et al. performed a transcriptional profile in three different leukemia cells lines (Jurkat, K562 and HL60) treated with 1 and 10 μM of adaphostin for 2 to 6 hours. The results demonstrated that adaphostin cytotoxicity in these cells is caused by the release of free iron resulting in redox alteration and cell death [115]. In a second study, proteomic analyses performed in myeloid leukemia cells treated with increasing concentrations of adaphostin resulted in upregulation of oxidative stress related proteins such as heat shock proteins, glutathione s-transferase, and superoxide dismutase [116]. Furthermore, studies in glioblastoma cells treated with adaphostin indicate that the antioxidant heme oxygenase-1 is upregulated [117].

Combinatory studies also have demonstrated the importance of ROS in the cytotoxic effect of adaphostin. A panel of leukemia cells was treated with adaphostin in combination with the proteasome inhibitor bortezomib, resulting in synergistic induction of apoptosis. This synergy was associated to elevated levels of ROS, downregulation of the Raf/MEK/ERK pathway and JNK activation. All these events were attenuated when cells were treated with the antioxidant NAC, demonstrating that oxidative stress plays an important role in the synergistic mechanism [118]. This combination was also tested in BCR/ABL positive cells resistant to imatinib resulting in synergistic induction of apoptosis in which potentiation of ROS was observed. Again, NAC blocked the synergistic effect implicating oxidative stress as a mechanism by which this combination overcomes imatinib resistance [118]. Taken together, these observations indicate that adaphostin is a redox-modulatory agent.

Two different studies of adaphostin's effect *in vivo* have been reported. One study showed that adaphostin increased survival in an orthotopic glioblastoma mouse model [119]. The second study, a report on the pharmacokinetics of adaphostin, showed that adaphostin was stable in human plasma [120]. Collectively, this data points to adaphostin as a good candidate to study in combination with HDACi.

1.5 Study Rationale, Hypothesis and Specific Aims

Despite advances in the treatment of pediatric leukemia, 20% of children die due to relapsed or refractory disease. Furthermore, survivors have an increased risk of death compared to age matched cohorts due to late effects of their therapy. Identification and development of new therapeutic strategies with specific and selective antileukemia activity are of utmost importance for these patients. Histone deacetylase inhibitors (HDACi), have shown to be effective in the treatment of hematological malignancies. The major accepted mechanism of action for these drugs is through hyperacetylation of histones. Interestingly, several studies have indicated that HDACi cause oxidative stress, which contributes to the cytotoxicity of these drugs [87]. Combining agents that cause further oxidative stress might enhance the efficacy of HDACi for the treatment of leukemia. The tyrphostin inhibitor adaphostin is one such agent. Adaphostin was originally identified as a tyrosine kinase inhibitor, but subsequent studies defined oxidative stress as its primary mechanism of action. Furthermore, adaphostin demonstrates selectivity for leukemia cells as compared to normal lymphocytes [113]. The present study investigates the efficacy and the redox mechanism of HDACi in combination with adaphostin in leukemia models.

The hypothesis for this study is that **HDACi in combination with adaphostin will synergize, causing oxidative stress and cell death through the activation of the apoptotic mitochondrial pathway.**

To test this hypothesis the following specific aims were conducted:

- **Specific Aim 1:** To determine the mechanism by which HDACi and adaphostin synergize to induce apoptosis.
- **Specific Aim 2:** To determine how ROS is generated by the combination and its role in the induction of apoptosis.
- **Specific Aim 3:** To determine how specific HDACi alter the oxidative environment to promote cell death**.**

Understanding the mechanism involved in the synergy between HDACi and adaphostin will advance scientific knowledge of how the action of HDACi could be augmented in leukemia models. Moreover, this information could be used for the development of effective clinical trials combining HDACi with other anticancer agents.

CHAPTER 2: MATERIALS AND METHODS

2.1 Cell lines

Jurkat and Molt-4 cells, representative of human T-acute lymphocytic leukemia (T-ALL) and I9.2 (caspase-8 deficient Jurkat cells) were acquired from American Type Culture Collection (ATCC; Manassas, VA). All cell lines were validated by short tandem repeats (STR) DNA fingerprinting by the MD Anderson characterized cell line core (CCSG) laboratory. All cells were grown in a humidified incubator with 5% CO_2 at 37^{o} C. Jurkat and Molt-4 were cultured in RPMI 1640 with 10% (Jurkat) or 20% (Molt-4) (v/v) heat-inactivation fetal bovine serum (FBS; Hyclone, Logan, UT), 2 mM L-glutamine, 100 U/mL penicillin, and 100 □g/mL streptomycin (Sigma St. Louis, MO). I9.2 cells were cultured in RPMI 1640 with HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic), sodium pyruvate and L-glutamine (ATCC; Manassas, VA) supplemented with 15% FBS and 100 U/mL penicillin, and 100 □g/mL streptomycin (Sigma St. Louis, MO).

2.2 Chemicals and antibodies

Adaphostin was provided by the NCI/NIH Developmental Therapeutics Program (Bethesda, MD). Vorinostat was purchased from Cayman Chemicals (Ann Arbor, MI) and entinostat was provided by Syndax (Waltham, MA). Propidium iodide (PI), N-Acetyl-L-cysteine (NAC), thioredoxin reductase from rat liver, NADPH and insulin from bovine pancreas were purchased from Sigma (St. Louis, MO). Dye for the detection of intracellular superoxide (dihydroethium - HE) was purchased from Invitrogen (Grand Island, NY). Tetramethylrhodamine ethyl ester (TMRE) was purchased from Molecular Probes (Eugene, OR). Caspase-3 substrate, DEVD-amc and the caspase inhibitors zVAD-fmk, DEVD-fmk, LEHD-fmk and IETD-fmk were purchased from Enzo Life

Sciences (Farmingdale, NY). Antibodies were purchased for polyclonal anti-acetyl-histone H3 and anti-histone H3 (Abcam, Inc., Cambridge, MA), monoclonal anti-thioredoxin (BD Biosciences Pharmigen, San Diego, CA) and anti-acetylated lysine (Cell Signaling, Danvers, MA).

2.3 DNA fragmentation and synergy analysis

Sub-diploid population was measured as an indicator of apoptosis using propidium iodide (PI) staining followed by flow cytometric analysis. After 24 h of incubation with desired treatment, cells were centrifuged, washed with PBS and suspended in 500 μL of PI solution (50 □g/mL PI, 0.1 % Triton X-100, and 0.1% sodium citrate in PBS) for a minimum of 3 h. Samples were assessed by flow cytometry on the FL-3 channel (FACSCalibur, Becton, Dickinson, Franklin Lakes NJ). CellQuest software (BD Bioscience, Franklin Lakes NJ) was used for analysis of the data. After three different experiments, synergy was calculated using isobologram analysis based on the Chou and Talalay method [121] with Calcusyn software (Biosoft, Ferguson, MO).

2.4 Detection of changes in mitochondrial membrane potential

Changes in mitochondrial membrane potential were measured using tetramethylrhodamine ethyl ester (TMRE) staining. TMRE is a cell permeable dye with positive charge, which is reduced by the interaction with active positively charged mitochondria. Increase in reduced TMRE is indicative of changes in mitochondrial membrane potential. After treatment with synergistic concentrations, cells were centrifuged, washed with PBS, and incubated with 25 nM TMRE in 10 mM HEPES,

150 mM NaCl, 5 mM KCl, 1 mM MgCl, and 1.8 mM CaCl, pH 7.4 in a volume of 1 mL for 30 min at 37℃ in the dark. After 30 min, cells were washed in PBS and fluorescence intensity was analyzed by flow cytometry on the FL-2 channel and analyzed by CellQuest software.

2.5 Detection of intracellular superoxide

The intracellular superoxide level was measured using the cell-permeable dihydroethidium (HE) dye followed by flow cytometry analysis. Briefly, cells were centrifuged and suspended in 1 mL of PBS containing 330 nM HE. The samples were incubated for 30 min in the dark at 37^{o} C. Cells were centrifuged and washed with PBS and re-suspended in 500 μl of PBS. Fluorescence intensity was assessed by flow cytometer on the FL-3 channel and analyzed by CellQuest software.

2.6 Caspase-3-like activity assay

After treatment with the synergistic concentrations, cells were centrifuged, washed, then suspended in 150 μl PBS and lysed by freezing and thawing once on dry ice. Triplicates of 50 μl of lysate were loaded in a 96-well plate and 150μl of 50 □M DEVD-amc in DEVD buffer (10% sucrose, 0.001% IGEPAL, 0.1% CHAPS, 5 mM HEPES, pH 7.25) were added to each well. The release of fluorescence (amc) generated from the cleavage of DEVD-amc was measured using a spectrofluorometer (SpectraMax Gemini EM, Molecular Devices, Sunnyvale, CA) with an excitation of 355 nm and emission of 460 nm.

2.7 Acid extraction of histones

Purification of histones was performed as described previously [122]. Briefly, after drug treatment, cells were harvested and nuclei were isolated by lysis of cells in a hypotonic buffer containing 10 mM Tris-HCl, pH 8.0, 1 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT. Protease and phosphatase inhibitors at a final concentration of 1 mM were added immediately prior to lysis. Lysates were rotated for 30 min at 4°C, and then centrifuged for 10 min at 10,000 rpm at 4°C. Supernatant was removed and isolated nuclei were suspended in 400 μl of 0.4 N H_2SO_4 and rotated for 30 min or overnight at 4°C. Nuclei lysates were centrifuged for 10 min at 12,500 rpm at 4°C to remove the nuclear debris. Supernatant was transferred to a new eppendorf tube and 132 μl of trichloroacetic acid (TCA) was added and incubated on ice for 30 min, then centrifuged for 10 min at 12,500 rpm at 4°C. Supernatant was removed and the histone pellet was washed with ice-cold acetone and centrifuged for 5 min at 12,500 rpm at 4°C. Finally the acetone was removed and histone pellets were dissolved in 100 μl of sterile water.

2.8 Immunoprecipitation

After treatment, cells were resuspended in lysis buffer (1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 20 mM sodium phosphate, pH 7.4). Aliquots of protein lysates (1000 μg) were immunoprecipitated overnight with 1 μg/mL of Trx antibody. Protein G agarose beads were added to each sample and rotated for a minimum of 2 h, then washed with lysis buffer and centrifuged three times. Samples were loaded onto a 15% sodium dodecyl sulfate (SDS)-polyacrylamide gel, transferred to nitrocellulose membrane, and blocked for 1 h with 5% nonfat dry milk in Tris-Buffered Saline Tween-20 (TBS-T) (0.05% Tween-20). Membranes were probed overnight at 4°C with 1:1000

dilution of primary antibody (acetylated-lysine) in 5% milk in TBS-T. The bound antibodies were detected using enhanced chemiluminescence, ECL plus Western blotting detection system (Amersham Bioscience, UK limited, Little Chalfont Buckinghamshire, England).

2.9 Quantification of cellular glutathione (GSH) levels

After 24 h of treatment, cells were harvested and equal numbers of cells were aliquoted to new tubes and 50 µM of monochlorobimane (mBCL) was added to each sample, except for the unstained control. mBCL is a cell permeable probe, which react with the thiol groups in GSH resulting in release of a fluorescent signal. Cells were incubated with the mBCL for 15 minutes at 37℃. After incubation, the reaction was stopped by adding trichloroacetic acid to a final concentration of 5%. Samples were collected and an equal volume of methylene chloride was added, followed by centrifugation at 3500 RPM for 2 minutes. After centrifugation, 200 µl from the aqueous phase was collected in a well of a white opaque 96 well plate. Fluorescence was measured using a microplate reader (Spectra Gemini EM, Molecular Devices, Inc. Sunnyvale CA) with excitation 398 nm and emission of 488 nm. GSH level was calculated using a standard curve with increasing GSH concentration that was prepared in parallel with the samples.

2.10 Thioredoxin Activity

Trx activity was determined using a spectrophotometric end-point insulin assay, where insulin is used as a substrate to measure the thiol formation in the reaction [123]. Briefly, after treatment with the synergistic concentrations, cells were centrifuged, washed with PBS and homogenized in cold buffer (50 mM potassium

phosphate, pH 7.4, containing 1 mM EDTA) followed by three cycles of freezing and thaws. Thirty μg of sample protein were incubated with 85 mM HEPES, 0.3 mM insulin, 660 μM NADPH, 3 mM EDTA and 0.1 unit of Trx reductase (TrxR) at 37°C for 60 min. After incubation, the reaction was stopped by adding 500 μl of 6 M guanidine hydrochloride with 4 mg/mL of 5,5′-dithio-bis (2-nitrobenzioic acid). Absorbance at 412 nm was measured using a SpectraMax Gemini EM spectrophotometer. For each sample, a blank containing all reagents, except TrxR, was used, and absorbance of the blank was subtracted from that of the sample with TrxR.

2.11 Oxidative stress and antioxidant defense PCR array

Total RNA was isolated from cells using the RNeasy Mini Kit (Qiagen, Valencia, MA). The concentration and quality of the RNA were measured using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific Inc., Walthaman, MA). For each sample, 0.5 μg of RNA was used to synthesize cDNA using the RT^2 First Strand Kit (Qiagen, Valencia, MA). Real-time PCR was performed according to the manufacturer's protocol conditions (Qiagen, Valencia, MA).

2.12 *In vivo* experiments

Female NOD/SCID, NOD-SCID-IL-2gnull and NOD-Cg-Rag1 mice purchased from the National Cancer Institute (Bethesda, MD) were used. All animals used for *in vivo* experiments were housed in standard cages, at three to four mice per cage and provided with food and water. Animal experiments were performed in accordance with the MD Anderson Institutional Animal Care and Use Committee and approved by the American Association for Laboratory Animal Science (AALAS). Mice were injected

intravenously (IV) with $1x10^5$ Molt-4 cells (representative of T-ALL). One week after cell injection, mice bearing leukemia cells were divided into four groups (3 mice per each group). Each mouse was injected intraperitoneally (IP) with vehicle, single drugs alone or drugs in combination. Dosing was done thrice weekly. To determine treatment efficacy, we conducted complete blood cell counts using approximately 25 µl of mouse blood on a weekly basis. Lymphocytes were isolated adding 1 mL of ACK (Ammoniun-Chloride-Potassium) lysing buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) to blood. Then samples were mixed followed by centrifugation at 12,000 RPM for 3 minutes. After centrifugation, supernatant was carefully aspirated and cell pellet was suspended in 50 µl of PBS. Isolated lymphocytes from mice were stained using 10 µl APC anti-human CD45 antibody (Biolegend Inc. San Diego, CA) in 50 µl of PBS containing cells, followed by flow cytometery and analysis using CellQuest software.

2.13 Statistical analysis

For each condition, at least three experiments were performed, and the results are presented as the mean ± standard deviation (SD). Statistical differences between conditions were analyzed using independent, two-tailed *t*-tests (Microsoft Excel software, Redmond, WA). A p-value < 0.05 was considered to be statistically significant. To determine synergy, Calcusyn software (Biosoft, Ferguson, MO) was used to calculate combination index (CI) values with isobologram analysis using the Chou and Talalay method [121]. A CI value less than 1.0 indicates synergy, equal to 1.0 indicates an additive effect and more than 1.0 represents an antagonist interaction.

CHAPTER 3: RESULTS

3.1 HDACi (vorinostat and entinostat) synergize with adaphostin to induce DNA fragmentation in ALL cells.

Previous studies have demonstrated that HDACi work better in combination than as a single agent in different types of cancer including hematological malignancies. In addition, data suggests that one mechanism of action for HDACi is through the induction of oxidative stress. To test if combining HDACi with a ROS generating agent would augment HDACi efficacy, we treated two different types of ALL cell lines with HDACi and adaphostin (a ROS generating agent). Jurkat and Molt-4 cells were treated with a range of HDACi (vorinostat or entinostat) and adaphostin concentrations (100 nM – 2.5 μM), incubated for 24 h and DNA fragmentation was determined by flow cytometry. Figure 11 shows an increase in DNA fragmentation when Jurkat and Molt-4 cells were treated with increasing concentrations of the single agents. DNA fragmentation was significantly enhanced in Jurkat cells when treated with increased concentrations of adaphostin (0.5 and 0.75 μM) with 0.75 μM of vorinostat (63.5% and 71.8%, respectively; $p < 0.05$) or adaphostin (0.5, 0.6 and 0.75 μM) with 2.5 μM of entinostat (68.3%, 78.7 and 83.6%, respectively; $p < 0.05$). In Molt-4 cells, a significant increase in DNA fragmentation was observed when 1.5 or 2.5 μM adaphostin was combined with 1.5 μM vorinostat (70.5% and 78.3%, respectively; $p < 0.05$) or 2.5 μM adaphostin with 2.5 μM of entinostat (76.3%; $p < 0.05$).

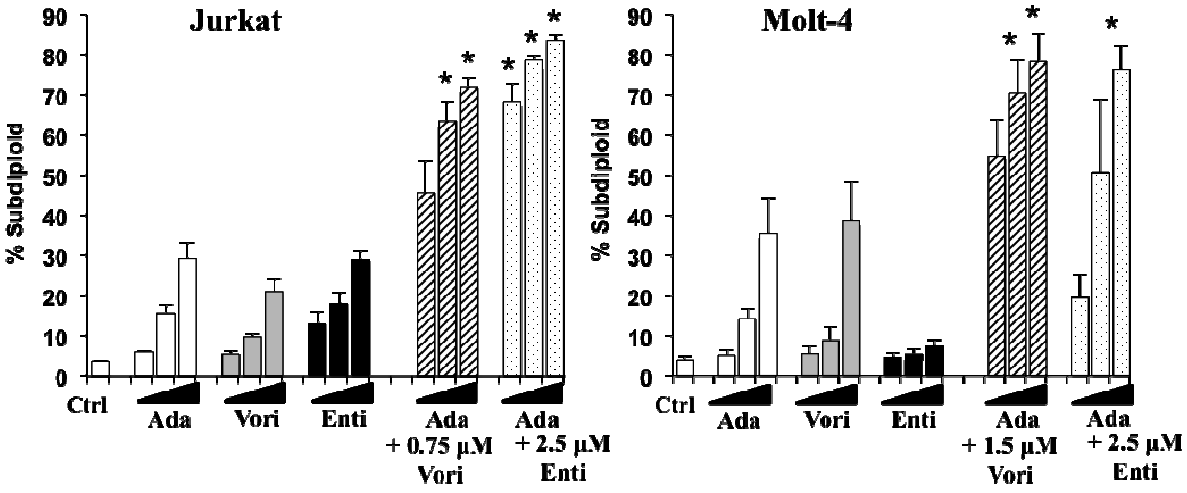
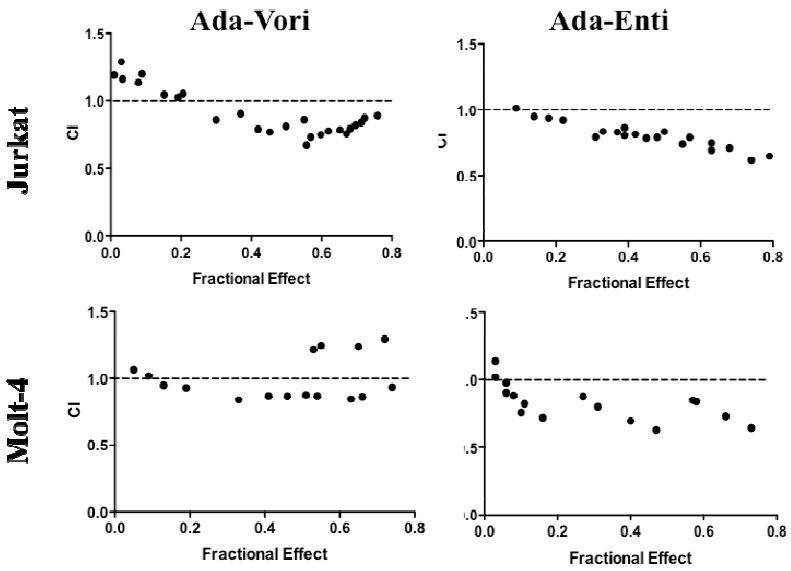


Figure 11. Combination of HDACi (vorinostat or entinostat) with adaphostin induces DNA fragmentation. 5 x 10^5 Jurkat cells (left panel) and Molt-4 cells (right panel) were treated with increasing concentrations (0.1-2.5 µM) of adaphostin (Ada), vorinostat (Vori), entinostat (Enti) or the combination of Ada-Vori or Ada-Enti for 24 h. (Drug concentration for Jurkat cells were 0.25, 0.5 and 0.75 µM Ada or Vori; 1, 1.5 and 2.5 µM Enti. For Molt-4 0.75, 1.5 and 2.5 µM Ada; 0.5, 0.75 and 1.5 µM Vori; 0.75, 1.5 and 2.5 µM Enti). DNA fragmentation (% Subdiploid) was assessed by PI staining following flow cytometry. Error bars represent the means ± S.D. of three independent experiments. *p < 0.05 as compared with either ada, vori or enti treatment alone.

Synergy analysis using the Chou and Talalay method [121], demonstrates that adaphostin synergizes with both vorinostat or entinostat in Jurkat and Molt-4 cells. A combination index (CI) value less than 1.0 indicates synergism whereas a CI value equal to 1.0 or more than 1.0 indicates an additive or antagonist interaction respectively. Representations of different synergistic combinations are presented in Figure 12.



Jurkat

Ada (μM)	Vori (μM)	Enti (μM)	CI
0.1	1	–	0.76
0.25	0.75	–	0.79
0.5	0.5	–	0.67
0.75	0.75	–	0.79
0.25	–	2.5	0.86
0.5	–	2.5	0.69
0.6	–	2.5	0.61
0.75	–	2.5	0.64

Molt-4

Ada (μM)	Vori (μM)	Enti (μM)	CI
0.5	1.5	–	0.86
0.75	1.5	–	0.87
1.5	1.5	–	0.86
2.5	1.5	–	0.93
0.5	–	2.5	0.75
0.75	–	2.5	0.72
1.5	–	2.5	0.62
2.5	–	2.5	0.642

Figure 12. HDACi (vorinostat or entinostat), synergize with adaphostin to induce DNA fragmentation. Synergy was calculated using isobologram analysis. A combination index (CI) value < 1.0 indicates synergism. Tables display some of the synergistic combinations in Jurkat and Molt-4 as indicated by the CI values.

To test if the combination of HDACi and adaphostin has any toxic effects in normal cells, peripheral blood mononuclear cells (PBMC) from healthy donors were isolated and treated with the synergistic combinations of 0.5 μM adaphostin plus 0.5 μM vorinostat or 0.75 μM adaphostin plus 2.5 μM entinostat. Cells were harvested after 24 h of incubation and cell viability was measured by trypan blue exclusion. Figure 13 shows that neither combination affected the viability of PBMC (Figure 13). In contrast, Jurkat cells treated with both combinations show a decrease in cell viability demonstrating specificity for leukemic cells.

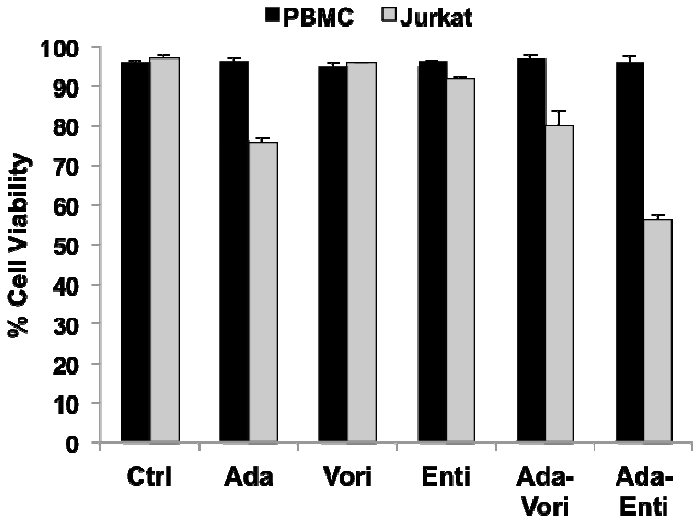


Figure 13. The combination of HDACi and adaphostin do not affect the cell viability of healthy peripheral blood mononuclear cells (PBMC). 5 x 10^5 PBMC or Jurkat cells were treated with 0.75 μM Ada, 0.5 μM Vori, 2.5 μM Enti and the synergistic combinations of 0.5 μM Ada plus 0.5 μM Vori or 0.75 μM Ada plus 2.5 μM Enti. After 24 h treatment, cell viability was measured by trypan blue exclusion. Error bars represent the means ± S.D. of three independent experiments.

To elucidate the mechanism of synergy, the subsequent experiments were performed in Jurkat cells with specific synergistic drug concentrations. The synergistic concentrations were selected based on the CI values (Figure 12). We decided to select two combinations that resulted in the low CI value of 0.6. These combinations were 0.5 μM of adaphostin in combination with 0.5 μM of vorinostat and 0.75 μM of adaphostin with 2.5 μM entinostat.

Since HDACi alone are known to induce acetylation of histones, we tested whether the synergistic combinations have any further effect on the acetylation of histones. Jurkat cells were treated with the synergistic combinations of adaphostin plus vorinostat or adaphostin plus entinostat and harvested at 24 h. Acid extraction of histones was performed and levels of total and acetylated histone H3 were measured by Western blot. As expected, increases in acetylation of histone H3 were observed in cells treated with either HDACi (vorinostat or entinostat). However, no further changes in acetylation were observed with either of the synergistic combinations (Figure 14).

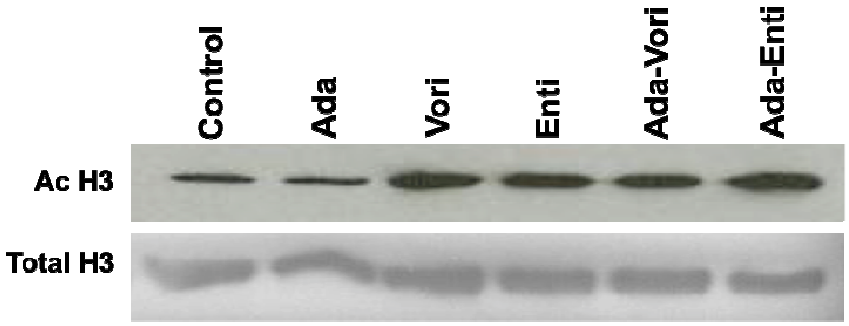


Figure 14. Synergistic combinations do not affect the acetylation of histone H3 induced by HDACi. 5 x 10^6 Jurkat cells were treated with the synergistic combinations of 0.5 μM Ada and 0.5 μM Vori or 0.75 μM Ada and 2.5 μM Enti. After 24 h of incubation cells were harvested and acid extraction of histones was performed. Acetylated histone H3 and total histone H3 were measured bv Western blot.

3.2 Induction of DNA fragmentation by HDACi and adaphostin is caspase-dependent.

Previous results demonstrate that the combination of HDACi and vorinostat synergize to induce DNA fragmentation in ALL cells. Since DNA fragmentation is a hallmark of apoptosis, we next investigated if a caspase-dependent apoptotic pathway was activated. In order to test if caspase-3 is activated and specifically at what time it is activated, we measured caspase-3-like activity in cells treated with the drug combinations at different time points. Jurkat cells were treated with the synergistic combinations of adaphostin plus vorinostat or adaphostin plus entinostat and harvested at different time points (2, 4, 8, 12, 16 and 24 h). Caspase-3-like activity was measured using DEVD-amc as a substrate. Cleavage of this substrate by caspase-3 liberates amc, creating a fluorescent signal, which was read on a spectrofluorometer. Figure 15 shows that both combinations increased caspase-3-like activity in a time dependent manner. Significant changes were observed at 12 h when compared to control (p-value < 0.05).

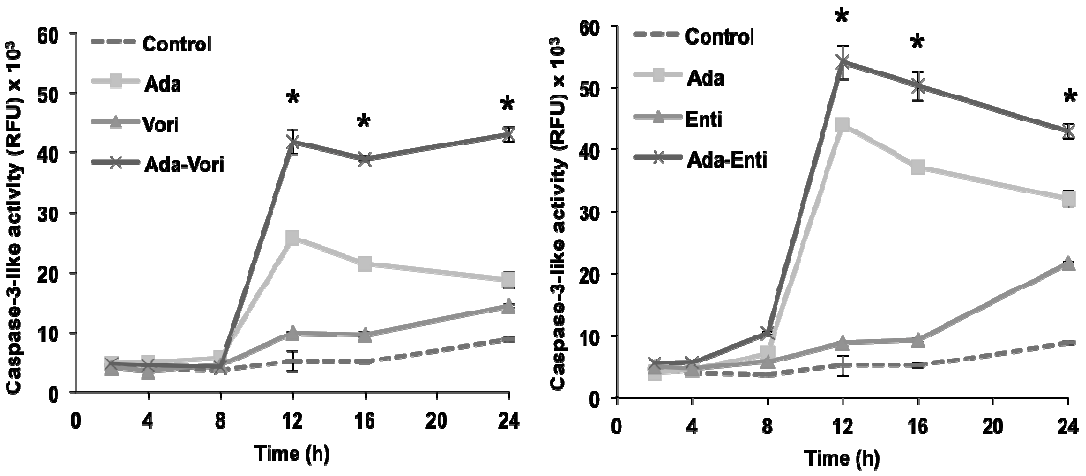


Figure 15. The combination of HDACi and adaphostin induce caspase-3 activation in a time dependent manner. 2 x 10^6 Jurkat cells were treated with the synergistic combinations of 0.5 μM Ada and 0.5 μM Vori or 0.75 μM Ada and 2.5 μM Enti. Caspase-3 activity was assessed by measuring the fluorescent intensity released from the cleavage of the fluorogenic substrate DEVD-amc at different time points (2 – 24 h). Error bars represent the means ± S.D. of two independent experiments. *p < 0.05 as compared with control or either agent alone.

To validate the previous results and to determine which specific apoptotic pathway is activated, cells were pre-treated with either 20 μM zVAD-fmk (pan caspase inhibitor), 25 μM IETD-fmk (caspase-8 inhibitor), 25 μM LEHD-fmk (caspase-9 inhibitor) or 25 μM DEVD-fmk (caspase-3 inhibitor) for 30 min and then treated with the synergistic concentrations of HDACi and adaphostin for 24 h. After 24 h, the cells were stained with PI and DNA fragmentation was measured by flow cytometry. As shown in Figure 16, apoptotic DNA fragmentation induced by the combinations was significantly reduced when caspase-8, -9 or -3 activity was blocked (*p*-value < 0.05). Comparing the fold changes in DNA fragmentation using each inhibitor, the caspase-9 inhibitor confers the stronger effect (7 fold change for ada-vori or 11 fold change for ada-enti) when compared to caspase-8 (3 fold change for ada-vori or 2 fold change for

ada-enti) or caspase-3 (3 fold change for ada-vori or 5 fold change for ada-enti) inhibitors.

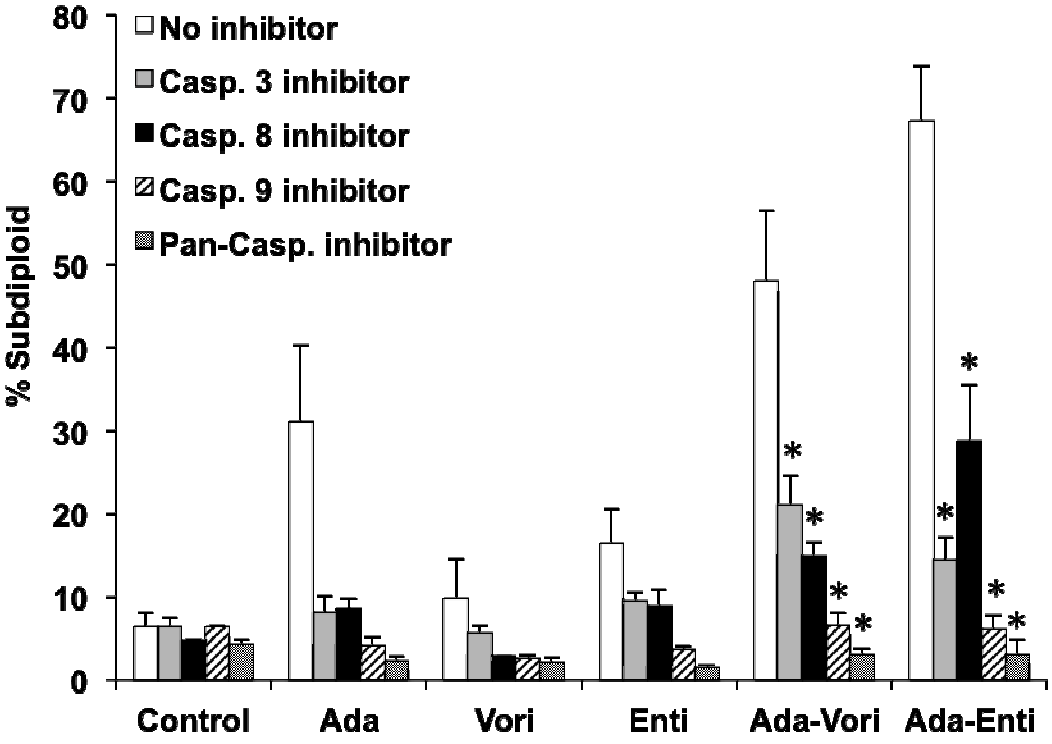


Figure 16. Induction of DNA fragmentation by HDACi and adaphostin synergy is caspase dependent. 5 x 10^5 Jurkat cells were pre-treated with either 20 µM pan-caspase, 25 µM caspase-3, caspase-8 or caspase-9 inhibitors for 30 min. After pre-treatment, the samples were treated with 0.5 µM Ada and 0.5 µM Vori or 0.75 µM Ada and 2.5 µM Enti for 24 h and dyed with PI. DNA fragmentation was assessed by flow cytometer. Error bars represent the means ± S.D. of three independent experiments. *$p < 0.05$ as compared with no inhibitor.

To better understand the role of caspase-8 in the induction of DNA fragmentation by the synergistic combinations, Jurkat variants that were caspase-8 deficient (I9.2 cells) were used. Parental Jurkat and I9.2 cells were treated with both synergistic combinations for 24 h. DNA fragmentation was measured by flow cytometry using PI staining. In both combinations, cells that lacked caspase-8 showed a moderate decrease in DNA fragmentation when compared to parental Jurkat cells (Figure 17 A-B). These data suggest that caspase-8 is not the principal caspase involved in the synergy between HDACi and adaphostin.

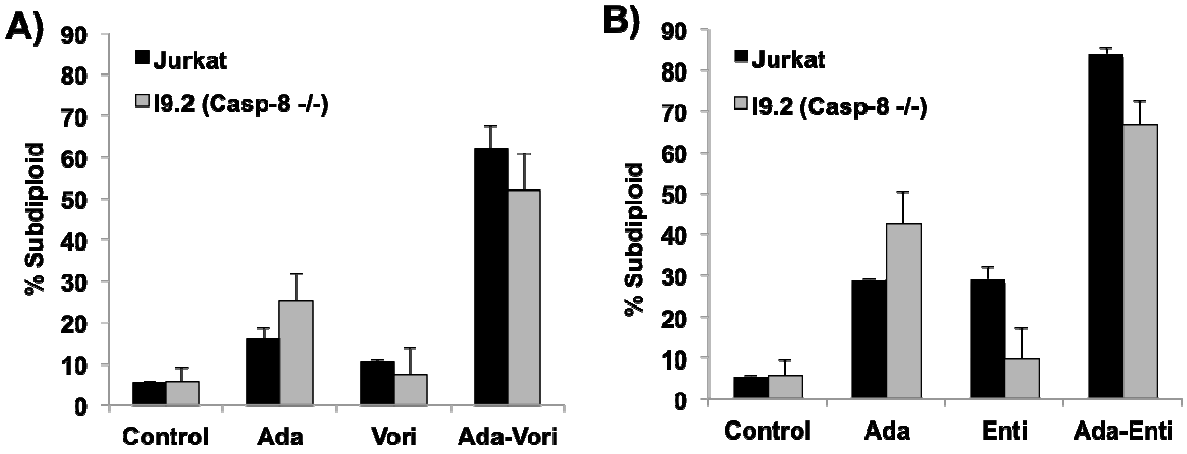


Figure 17. Caspase-8 is not required for the induction of DNA fragmentation by the combination of HDACi and adaphostin. 5×10^5 cells, either I9.2 (caspase-8 deficient) or parental Jurkat cells were treated with the synergistic combinations of 0.5 µM Ada and 0.5 µM Vori or 0.75 µM Ada and 2.5 µM Enti for 24 h. DNA fragmentation was measured by PI staining in cells treated with the synergistic concentration of A) Ada-Vori and B) Ada-Enti. Error bars represent the means ± S.D. of three independent experiments.

3.3 The combination of HDACi and adaphostin disrupts the mitochondrial membrane potential and potentiates superoxide levels.

Previous data generated using caspase inhibitors and caspase-8 deficient cells suggest that caspase-9 mediates the induction of apoptosis in cells treated with the combination of HDACi and adaphostin. Since caspase-9 can be activated by both apoptotic pathways (extrinsic and intrinsic), we tested if these drug combinations have any effect on the mitochondrial membrane potential. To determine if mitochondrial membrane potential is affected and specifically at what time this event occurs, Jurkat cells were treated with both combinations and harvested at different time points (2, 4, 8, 12, 16 and 24 h). Changes in mitochondrial membrane potential were measured by flow cytometry using TMRE staining. Significant loss of membrane potential was detected at 12 h and sustained through 24 h in both combinations (Figure 18 A-B).

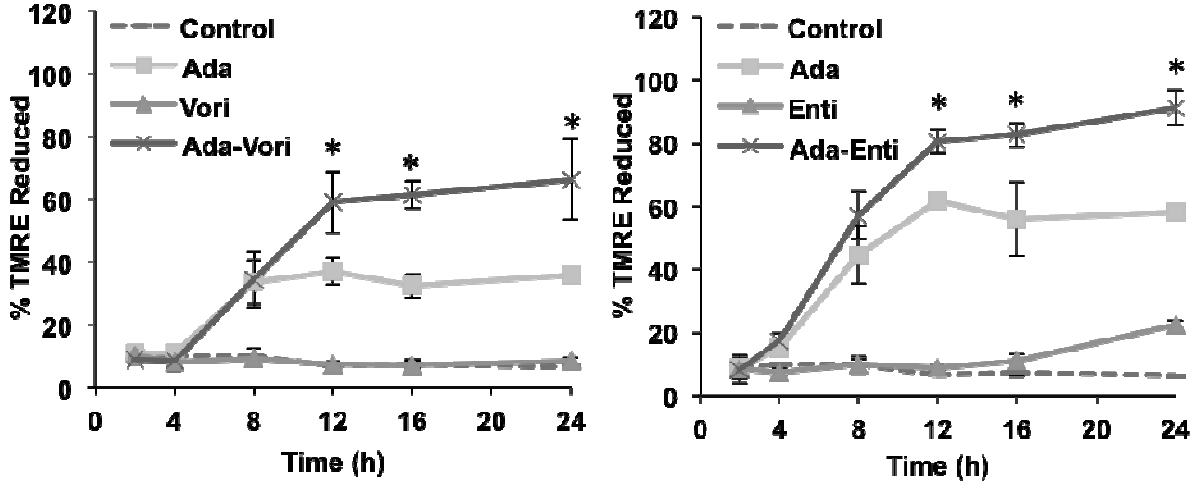


Figure 18. Combination of HDACi and adaphostin disrupts mitochondrial membrane potential starting at 12 h. 5 x 10^5 Jurkat cells were treated with the synergistic combinations of 0.5 µM Ada and 0.5 µM Vori or 0.75 µM Ada and 2.5 µM Enti. After treatment changes in mitochondrial membrane potential was measured by TMRE staining in A) Ada-Vori or B) Ada-Enti combinations at different time points (2-24 h). *p < 0.05 as compared with control.

To elucidate whether the loss of mitochondrial membrane potential precedes or follows caspase activation, cells were pre-treated with the pan-caspase inhibitor ZVAD followed by treatment with the synergistic concentrations. After 24 h, cells were harvested and mitochondrial membrane potential was measured. Figure 19 shows that ZVAD blocked the loss of membrane potential caused by both combinations. These results suggest that caspase activation is downstream of the changes in mitochondrial membrane potential.

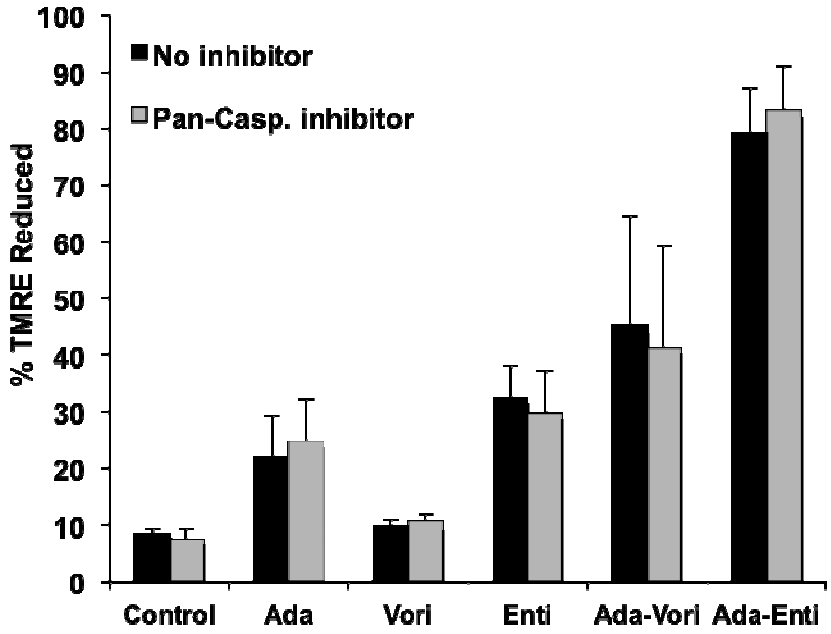


Figure 19. Caspase activation is downstream of loss of mitochondrial membrane potential. 5 x 10^5 Jurkat cells were pre-treated with 20 µM pan-caspase inhibitor for 30 min. After pre-treatment cells were treated with the synergistic combinations of 0.5 µM Ada and 0.5 µM Vori or 0.75 µM Ada and 2.5 µM Enti. After 24 h mitochondrial membrane potential was measured by TMRE staining. Error bars represent the means ± S.D. of three independent experiments.

Increased endogenous levels of ROS can promote mitochondrial membrane permeabilization resulting in the release of cytochrome c and activation of caspase-9. Since decreased mitochondrial membrane potential has been shown to promote an environment of oxidative stress, we sought to determine whether there was an increase in superoxide levels and, if so, at what specific time point these are generated. Cells were treated with the combinations and harvested at different time points (2, 4, 8, 12, 16 and 24 h) and superoxide levels were measured using HE staining. Results show that both combinations potentiate superoxide levels more than the single agents. Significant increases were observed at 16 h and continued increasing through 24 h (Figure 20 A-B). Based on the time course experiments, these results place the induction of oxidative stress downstream of the initial loss of mitochondrial membrane potential that started at 12 h.

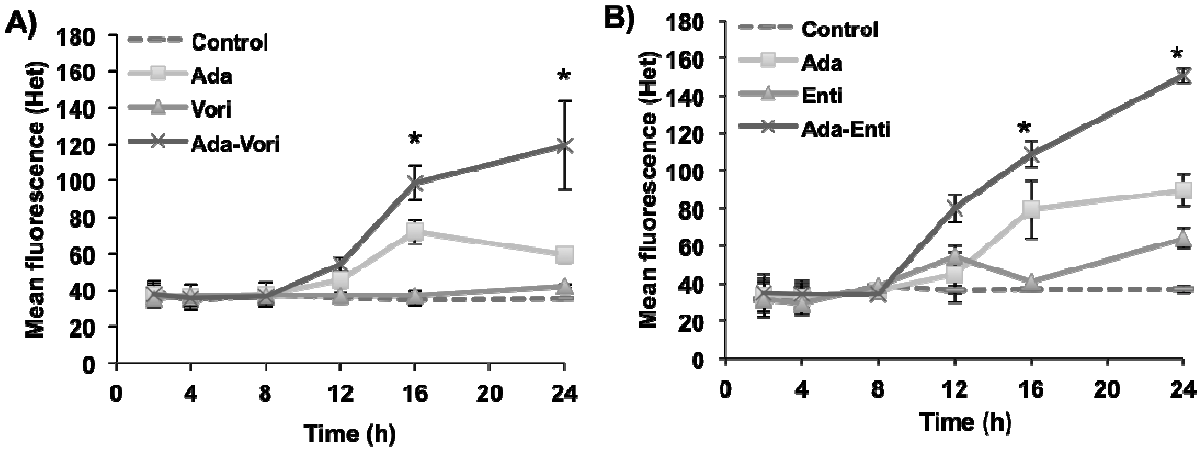


Figure 20. HDACi and adaphostin potentiate superoxide levels in a time dependent manner. 5 x 10^5 Jurkat cells were treated with the synergistic combinations of 0.5 µM Ada and 0.5 µM Vori or 0.75 µM Ada and 2.5 µM Enti. Superoxide levels were measured by HE staining in cells treated with the synergistic concentrations of A) Ada-Vori and B) Ada-Enti at different time points (2-24 h). Error bars represent the means ± S.D. of three independent experiments. *$p < 0.05$ as compared with control.

3.4 NAC blocks DNA fragmentation in cells treated with adaphostin and vorinostat, but not in cells treated with adaphostin and entinostat.

Previous results suggest that oxidative stress might play an important role in the mechanism of synergy between HDACi (vorinostat and entinostat) and adaphostin. In order to elucidate if ROS contributes to cell death, we used NAC as an antioxidant. Cells were pre-treated with 24 mM NAC for 30 min. Then cells were treated with the drug combinations. After 24 h of treatment superoxide levels, changes in the mitochondrial membrane potential, DNA fragmentation and caspase-3 activity were measured as previously described. As expected, NAC decreased the levels of superoxide in both combinations, demonstrating that NAC is blocking oxidative stress (Figure 21A). In addition, NAC was able to reverse the loss of mitochondrial membrane potential in both combinations, suggesting that production of oxidative stress precedes the loss of mitochondrial membrane potential (Figure 21B). Finally, to investigate the role of superoxide production in cell death, we measured DNA fragmentation and caspase-3 activity in cells pre-treated with NAC and then with the drug combinations. DNA fragmentation was decreased by NAC in cells treated with adaphostin plus vorinostat, but not in cells treated with adaphostin plus entinostat (Figure 21C). These results were confirmed when caspase-3 activation was measured in cells pre-treated with NAC followed by the drug combinations (Figure 21D). All together, these results suggest that at the synergistic concentrations, the combination of adaphostin plus vorinostat might have a different mechanism of inducing oxidative stress mediated-apoptosis compared to the combination of adaphostin plus entinostat.

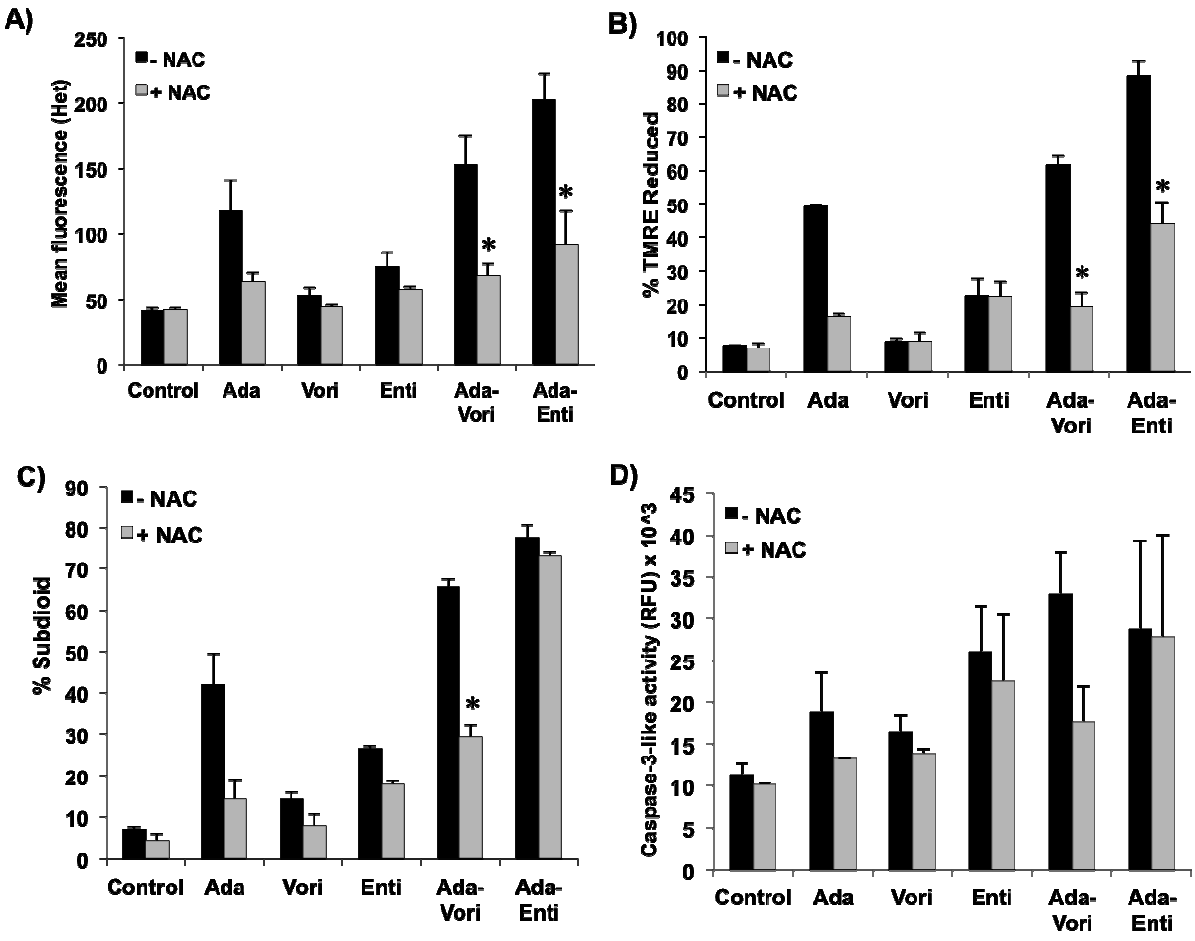


Figure 21. Superoxide levels precede the loss of mitochondrial membrane potential and NAC blocks cell death in cells treated with Ada-Vori but not Ada-Enti. Jurkat cells were pretreated with 24 mM NAC for 30 min, then treated with the synergistic combinations of 0.5 µM Ada and 0.5 µM Vori or 0.75 µM Ada and 2.5 µM Enti. After 24 h treatment, 5 x 10^5 cells were harvested to measure A) Superoxide levels, B) Mitochondrial membrane potential, C) DNA fragmentation and 2 x 10^6 cells were harvested to measure D) Caspase-3-like activity. Error bars represent the means ± S.D. of three independent experiments. *$p < 0.05$ as compared with no NAC.

3.5 Glutathione (GSH) & thioredoxin (Trx) are not affected by the synergistic combination of adaphostin and HDACi.

In order to elucidate the main differences between these two combinations we first assessed the involvement of GSH in the mechanism of synergy. GSH were measured in cells treated with the drug combinations. After 24 h of treatment, cells were harvested and GSH levels were measured using monochlorobromane as a substrate, which react with the thiol groups in GSH resulting in release of a fluorescent signal. Results show a two-fold decrease in GSH levels in cells treated with adaphostin and entinostat, and one-fold decrease in cells treated with adaphostin and vorinostat (Figure 22).

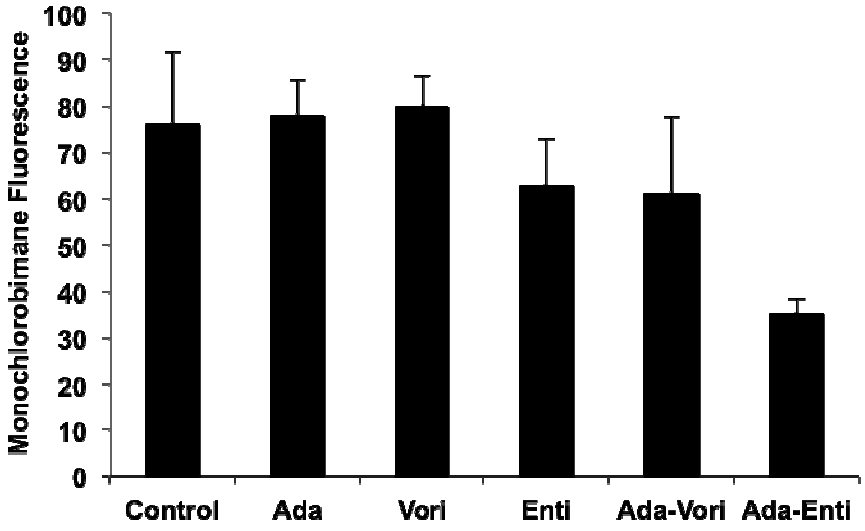


Figure 22. Glutathione (GSH) levels in cells treated with HDACi and adaphostin. 1 x 10^6 Jurkat cells were treated with the synergistic combinations of 0.5 µM Ada and 0.5 µM Vori or 0.75 µM Ada and 2.5 µM Enti. After 24 h treatment, GSH levels were measured using monochlorobimane as a substrate. Error bars represent the means ± S.D. of three independent experiments.

Since NAC not only alters the levels of GSH, but any cysteine-containing molecule, and to better elucidate the role of GSH in this mechanism, we used glutathione ethyl ester (GSHee) to directly address a role for GSH. GSHee is a soluble molecule, which enters in the cell and undergoes hydrolysis by intracellular esterases to be converted to GSH. Therefore, treatment with GSHee is commonly used to increase levels of GSH within the cells.

As expected, increases in GSH levels were observed in cells pre-treated with GSHee (Figure 23A). However GSHee did not block the previously observed loss of mitochondrial membrane potential or DNA fragmentation (Figure 23B-C). These results suggest that changes in GSH levels are not the main mechanism of action for the generation of oxidative stress in the present model.

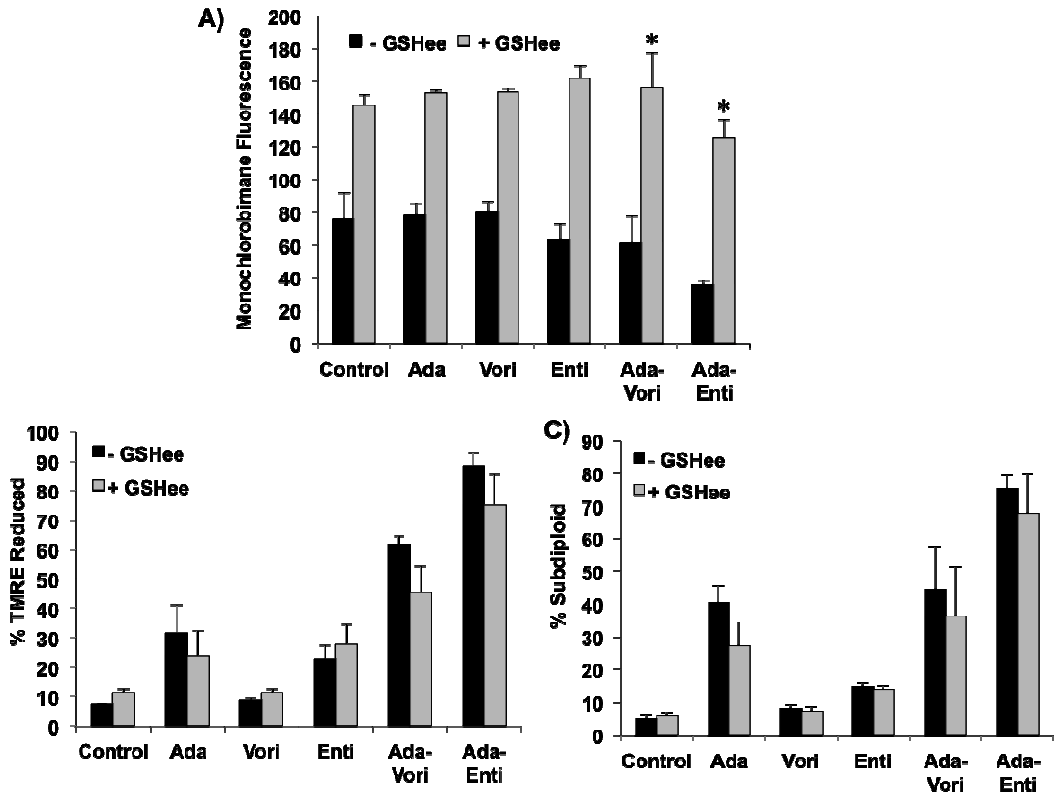


Figure 23. GSHee increases the levels of GSH but does not block the loss of mitochondrial membrane potential or cell death in cells treated with HDACi-Adaphostin. Jurkat cells were pre-treated with 1 mM GSHee for 30 min and then treated with the synergistic combinations of 0.5 µM Ada and 0.5 µM Vori or 0.75 µM Ada and 2.5 µM Enti. After 24 h treatment, 1 x 10^6 cells were harvested to measure A) GSH levels; and 5 x 10^5 cells were harvested to measure B) mitochondrial membrane potential and C) DNA fragmentation were measured as described previously. Error bars represent the means ± S.D. of three independent experiments. *$p < 0.05$ as compared with no GSHee.

Thioredoxin (Trx) is another cellular antioxidant and its activity has been demonstrated to be affected by HDACi. Vorinostat was found to decrease Trx activity by the upregulation of thioredoxin binding protein 2 (TBP-2) [92]. Furthermore, recent studies have shown that HDACi can acetylate Trx [62]. However, whether this acetylation affects Trx activity remains unknown. We decided to investigate if acetylation of Trx plays an important role in the synergy between adaphostin and HDACi. First, we tested if the combination of HDACi and adaphostin induces Trx acetylation. Figure 18A shows that acetylation of Trx is induced in cells treated with higher concentrations of vorinostat (5 μM) but not at lower concentrations (0.5 μM vorinostat). However, Trx acetylation was observed in cells treated with 2.5 μM entinostat and this acetylation was further increased by the combination of 0.75 μM adaphostin and 2.5 μM entinostat (Figure 24A). In order to elucidate if the synergistic combinations affect Trx activity, we measured Trx activity in cells treated with either agent alone or in combination. No changes in Trx activity were observed after treatment with either of the combinations (Figure 24B).

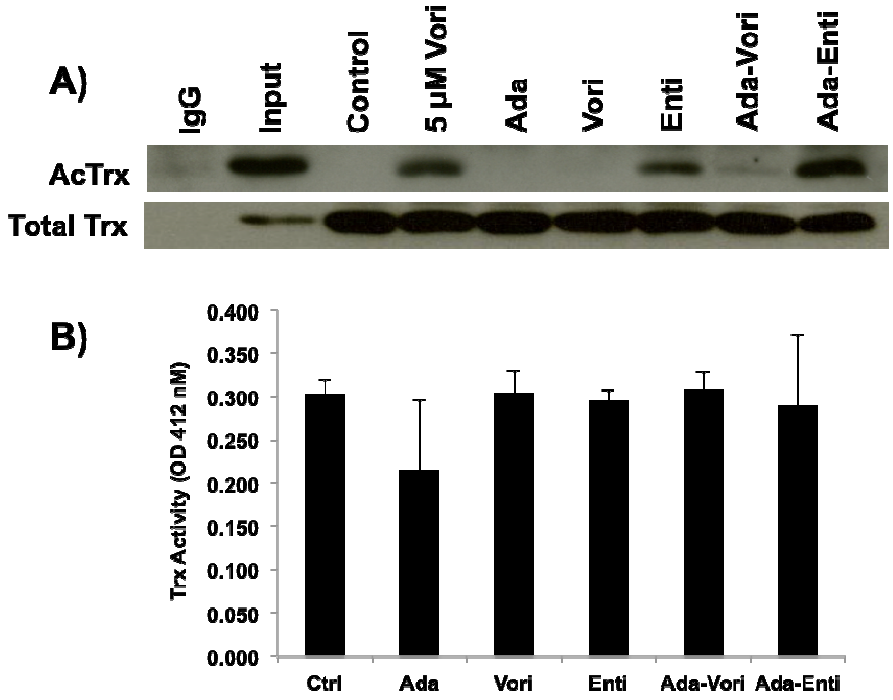


Figure 24. The combination of HDACi and adaphostin increases the acetylation of Trx but does not affect Trx activity. Jurkat cells were treated with the synergistic combinations of 0.5 µM Ada and 0.5 µM Vori or 0.75 µM Ada and 2.5 µM Enti. After 24 h of incubation 20 x 10^6 cells were harvested for the immunoprecipitation and 5 x 10^6 cells were harvested to measure Trx activity. A) Immunoprecipitation of Trx was performed and blotted for acetylated Trx. B) Trx activity was measured using a spectrophotometric end-point insulin assay. Error bars represent the means ± S.D. of three independent experiments.

3.6 HDACi and adaphostin combinations induce differential regulation of oxidative stress related genes.

Previous results demonstrated that NAC blocked the loss of mitochondrial potential and ROS levels, suggesting that oxidative stress plays an important role in the synergy between HDACi with adaphostin. However, NAC blocked DNA fragmentation in cells treated with adaphostin plus vorinostat but not in cells treated with adaphostin plus entinostat. To elucidate these differences we investigated the role of GSH and Trx. Results demonstrated that GSH and Trx are not major players in these differences. To better understand what factors are involved in the differences between the two combinations, we examined the expression of different genes involved in oxidative stress and antioxidant defense. Since we observed changes in superoxide levels starting at 16 h, we hypothesized that gene changes start earlier. For that reason, we decided to examine the changes in gene expression at 8 h. RNA was extracted from cells treated with the synergistic combinations for 8 h. cDNA was synthesized and a RT^2 profile PCR array for genes involved in oxidative stress and antioxidant defense was performed. Gene regulation was compared between cells not treated (control) and cells treated with either combination (Ada-Vori or Ada-Enti). Then these results were compared between each combination. We selected the genes with the major fold changes in gene regulation induced by Ada-Vori compared to Ada-Enti (Table 1). This data shows that genes involved in oxidative stress were differentially regulated between Ada-Vori and Ada-Enti combinations. In cells treated with Ada-Enti compared to Ada-Vori, there was up-regulation of aldehyde oxidase 1, glutathione peroxidase-2, lactoperoxidase, eosinophil peroxidase 2 and scavenger receptor class A. In cells treated with Ada-Vori compared to Ada-Enti, there were up-

regulations of glutathione peroxidase 6, peroxiredoxin 6 and down-regulation of myeloperoxidase and angiopoietin-like 7.

Gene	Ctrl vs Ada-Vori	Ctrl vs Ada-Enti
Eosinophil peroxidase	1.3195	2
Glutathione peroxidase 2	1.1487	2.1435
Lactoperoxidase	1	2.8284
Aldehyde oxidase 1	1.8661	5.6569
Scavenger receptor class A	1	4.2871
Glutathione peroxidase 6	2.639	1.1487
Peroxiredoxin 6	2	1.2311
Chemokine ligand 5	2.4623	7.4643
Metallothionein 3	36.7583	137.187
Glutathione peroxidase 5	2	12.1257
Angiopoietin-like 7	-27.8576	1.6245
Myeloperoxidase	-2.2974	1.2311

Table 1. Genes involved in oxidative stress are differentially regulated between Ada-Vori and Ada-Enti combinations. 5 x 10^6 Jurkat cells were treated with the synergistic combinations of 0.5 µM Ada and 0.5 µM Vori or 0.75 µM Ada and 2.5 µM Enti. After 8 h cells were harvested and RT^2 profile PCR array for genes involved in oxidative stress and antioxidant defense was performed. Numbers in red represent a gene up-regulation, numbers in blue represent a gene down-regulation and number in black represent no change.

3.7 In vivo efficacy of HDACi in combination with adaphostin.

Previous results demonstrate that two different HDACi synergize with adaphostin to induce oxidative stress mediated-apoptosis *in vitro*. To test if this combination works *in vivo*, we used an orthotopic mouse model. Since the U.S. Food and Drug Administration has not yet approved entinostat, we decided to use vorinostat for the *in vivo* experiments. Figure 25 shows the experimental design.

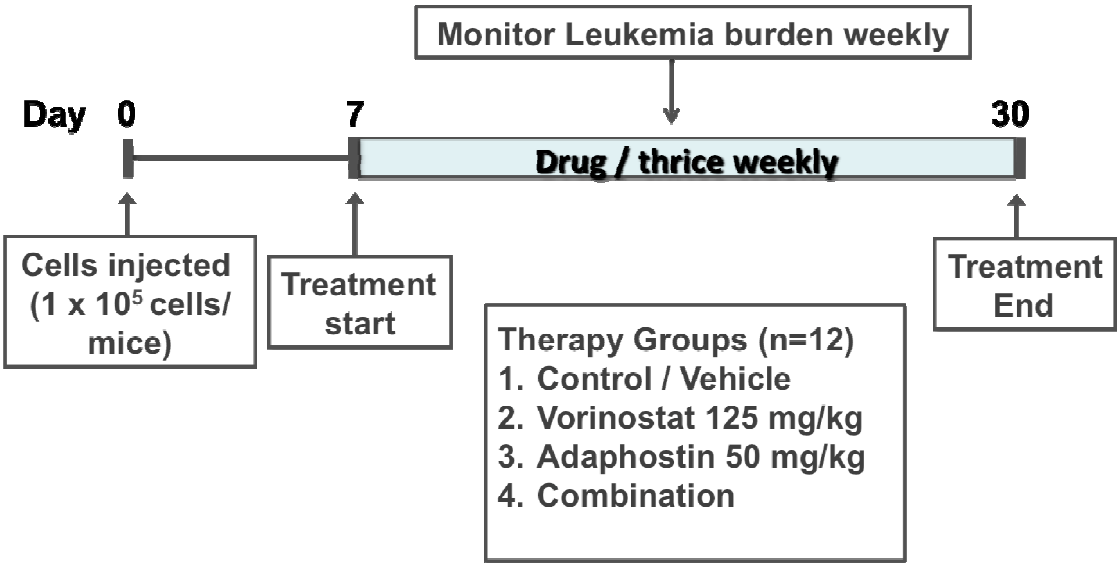


Figure 25. Schematic diagram of *in vivo* experimental design. NOD/SCID mice were injected intravenously with leukemia cells and one week after cell injection, treatment started. Drugs were administrated the same days thrice weekly. Leukemia burden was monitored weekly by measuring CD34+ cells present in the blood.

Twelve NOD-SCID-IL-2gnull mice were injected intravenously (IV) with $1x10^5$ Molt-4 cells (representative of T-ALL). One week after cell injection, mice bearing leukemia cells were divided into four groups (3 mice per group). We selected the administration routes and doses based on published data [124, 125]. Each mouse was injected intraperitoneally (IP) with vehicle, drugs alone or in combination. Group 1 was injected with vehicle (DMSO). Group 2 was injected with 125 mg/kg [124] vorinostat. Group 3 was injected with 50 mg/kg adaphostin [125]. Group 4 was injected with the drug combination (vorinostat and adaphostin). Drug dosing was done on the same days thrice weekly. To determine treatment efficacy we conducted complete blood cell counts using approximately 25 µl of mouse blood on a weekly basis. Lymphocytes were isolated using ACK lysing buffer. Isolated lymphocytes were stained using a fluorescence conjugated antibody (APC anti-human CD45) followed by flow cytometric analysis.

After the start of drug administration, the mice injected with 50 mg/kg of adaphostin died unexpectedly on day 11. We observed leukemic cell engraftment at week 3. One mouse from the group treated with vorinostat died on day 29 and the rest of the mice died around day 30-33 (Figure 26).

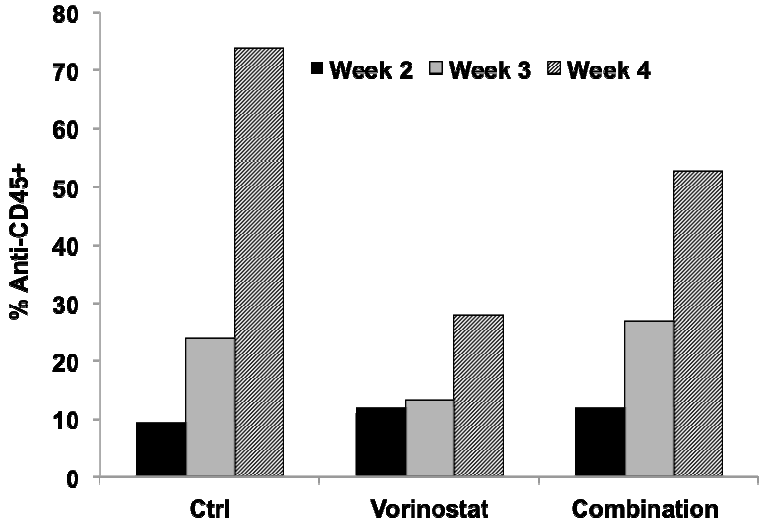


Figure 26. Molt-4 cells engraftment started at week 3. Twelve NOD-SCID-IL-2gnull mice were injected intravenous with $1x10^5$ Molt-4 cells. After one week of cell injection, 3 mice per group were treated thrice weekly with vehicle, 50 mg/kg adaphostin, 125 mg/kg vorinostat or the combination of adaphostin and vorinostat. Leukemia burden was monitored by bleeding and isolated lymphocytes from mouse were stained with anti-human CD45 and measured by flow cytometry. Each bar represents the average of 3 mice per group or treatment. No bars are presented in the group treated with adaphostin because mice died during week 1.

Mice treated with 50 mg/kg of adaphostin died during week 1, suggesting adaphostin toxicity. To find a less toxic dosage, we performed a second pilot by using NOD-SCID mice, decreasing the drug dosages and changing the treatment schedule. On this occasion we treated mice with lower doses of each drug daily instead of thrice a week. Mice were IP injected with 25 mg/kg adaphostin, 50 mg/kg vorinostat or the combination of vorinostat and adaphostin. After the second week of treatment, we observed abnormal growth in the abdominal area of mice treated with adaphostin and the combination. Two mice, one control treated with vehicle and one treated with adaphostin, were sacrificed and necropsies were performed.

Necropsies demonstrated gastrointestinal (GI) toxicity in mice treated with adaphostin (Figure 27). After these findings, we decided to decrease the dose of adaphostin from 25 mg/kg to 12.5 mg/kg.

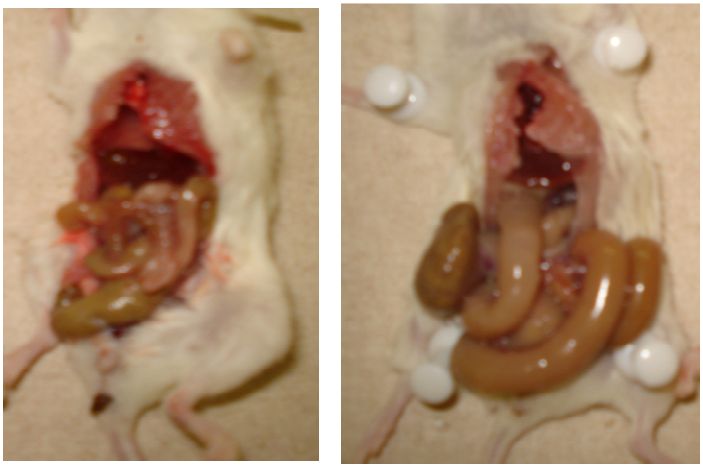


Figure 27. GI toxicity in mice treated with adaphostin. Necropsy of mouse treated with either vehicle (DMSO) or 25 mg/kg adaphostin showed an increase in the size of the GI track in mouse treated with adaphostin. In contrast mouse treated with vehicle showed normal GI track.

At week four we did not observe cell engraftment and we injected 5 x 10^5 Molt-4 cells into each of the remaining mice. We did not observe any cell engraftment at week 6 (Figure 28) and mice treated with adaphostin and the combination started to show GI toxicity again at week eight. The treatment was stopped at week nine.

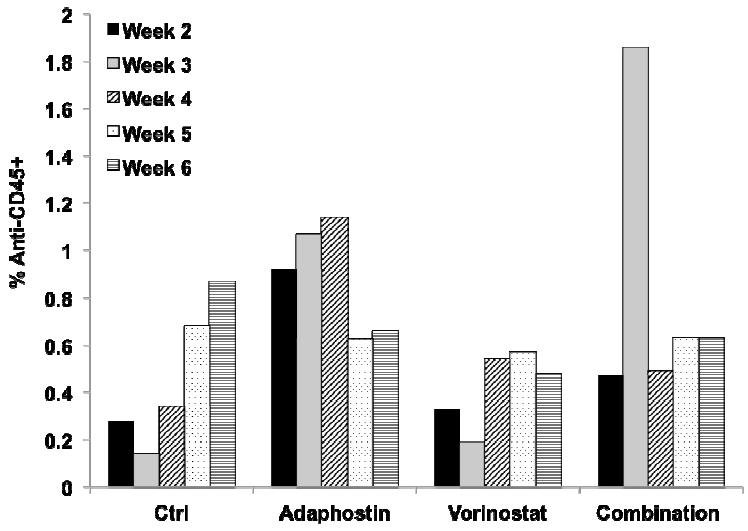


Figure 28. Molt-4 cell engraftment in NOD-SCID mice. NOD-SCID mice were injected intravenously with $1x10^5$ Molt-4 cells. After one week, mice were treated for five days weekly with vehicle, 25 mg/kg adaphostin, 50 mg/kg vorinostat or the combination of adaphostin and vorinostat. Leukemia burden was monitored by bleeding and isolated lymphocytes from mice were stained with anti-human CD45 and measured by flow cytometry. Each bar represents the average of 3 mice per group or treatment.

Since we observed GI toxicity in mice treated with adaphostin, we decided to perform toxicity tests to find the appropriate adaphostin dose and treatment schedule. As presented in table 2, six mice were treated with different doses and schedules. At week two, one mouse treated with 12.5 mg/kg adaphostin presented with sign of a tumor in the left leg. We performed a necropsy and we did not find any signs of GI toxicity or leukemia burden. The remaining mice were treated with their respective doses for a total of five weeks. After five weeks, no sign of GI toxicity was observed in any of the mice.

Mice	Adaphostin (mg/kg)	Schedule
1	12.5	5 days / week
2	6.25	5 days / week
3	3.125	5 days / week
4	8	5 days / week
5	8	3 days / week
6	8	2 days / week

Table 2. Adaphostin doses and schedule administration. Mice were injected IP with different doses of adaphostin using different schedules. After five weeks of treatment, mice were sacrificed and necropsy was performed.

Based on the toxicity test, we decide to perform a third pilot study. On this occasion, NOD-Cg-Rag1 mice were injected with 1 x 10^6 Molt-4 cells per mouse. After seven days mice were treated with vehicle, 50 mg/kg of vorinostat for 5 days/week, and/or 8 mg/kg of adaphostin for 3 day/week. After five weeks of treatment we did not observe GI toxicity, but we didn't see cell engraftment (Figure 29).

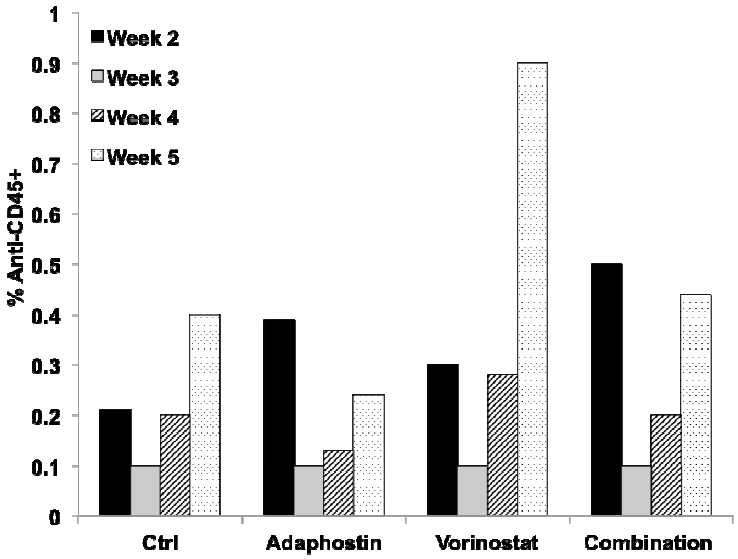


Figure 29. Molt-4 cell engraftment in NOD-Cg-Rag1 mice. NOD-Cg-Rag1 mice were injected intravenously with 1x10^6 Molt-4 cells. After one week, mice were treated with vehicle (5 days/week), 8 mg/kg adaphostin (3 days/week), 50 mg/kg vorinostat (5 days/week) or the combination of adaphostin and vorinostat. Leukemia burden was monitored by bleeding and isolated lymphocytes from mouse were stained with anti-human CD45 and measured by flow cytometry. Each bar represents the average of 3 mice per group or treatment.

CHAPTER 4: DISCUSSION

The main objective of this project is to understand how oxidative stress inducing agents might enhance the efficacy of HDACi for the treatment of ALL. For the first time, the ROS generating agent adaphostin was used in combination with two different HDACi (vorinostat and entinostat) in ALL cell lines. DNA fragmentation and combination index values demonstrated that HDACi, vorinostat and entinostat, synergize with adaphostin to induce apoptosis in ALL cells. Furthermore, the synergistic concentrations do not affect PBMC from healthy donors, demonstrating that this effect is selective for ALL cells. This synergy is mainly driven by the activation of the intrinsic apoptotic pathway, where time course analyses showed that perturbation of the mitochondrial membrane potential is the earliest event observed. Following this event, increasing levels of superoxide, caspase activation and ultimately cell death were observed.

Synergistic apoptosis induction by HDACi and adaphostin.

The observation that HDACi synergizes with adaphostin resulting in apoptosis is consistent with other studies combining HDACi with different drugs like topoisomerase inhibitors [126], nucleoside analogs [108], hypomethylating agents [127], proteasome inhibitors [50, 128] and tyrosine kinase inhibitors [129]. However, the mechanism by which these combinations induce apoptosis differs between combinations. These observations can be possibly explained by the pleiotropic effects of HDACi and the specificity of the cancer cell type. Furthermore, the mechanism of action of the agent with which the HDACi is combined also plays a role in which apoptotic mechanisms are activated.

In the present study, results of experiments using different caspase inhibitors (pan-caspase, caspase-8, -9, and -3 inhibitors) confirmed that the synergy observed between HDACi and adaphostin is through the induction of apoptosis. Both caspase-8 and -9 chemical inhibitors reduced DNA fragmentation in cells treated with the synergistic concentrations of HDACi and adaphostin (Figure 16). However, the caspase-9 inhibitor exerted a strong inhibition of cell death in both combinations. In addition, we found that both combinations induced loss of the mitochondrial membrane potential (Figure 18). Together, these results suggest that the main mechanism by which HDACi and adaphostin induce apoptosis is through the intrinsic pathway. These observations are consistent with other studies where HDACi has activated the intrinsic pathway in different types of cancer cells [130].

Zhang, et al. examined the effect of the HDACi, suberic bishydroxamate (SBHA), which is a derivate of vorinostat, in a panel of human melanoma cell lines. They found that HDACi activated the intrinsic apoptotic pathway. This activation was attributed to the down-regulation of antiapoptotic proteins XIAP, Bcl-XL and Mcl-1 and the up-regulation of proapoptotic genes like Bax, Bak and Bim. These changes resulted in mitochondrial disruption and consequent activation of caspase-3 [130]. In contrast to this study, we did not observe changes in the protein levels of XIAP (Appendix). This discrepancy can be explained by the use of the combination vorinostat or entinostat plus adaphostin instead of SBHA alone. Also the cancer type, since they used melanoma cells instead of leukemia cells might play an important role.

Other studies using vorinostat have also shown activation of the intrinsic apoptotic pathway by up-regulating Bim in lymphomas [89]. In addition, cleavage of Bid, perturbation of the mitochondria and release of cytochrome c also has been reported by others [87, 89]. In a similar way, entinostat induced the activation of the

intrinsic pathway in lymphoma cells by the down-regulation of Bcl-2 and Bcl-xL proteins. The combination of entinostat with two different Bcl-2 inhibitors significantly enhanced this effect [131]. Furthermore, different studies have demonstrated that overexpression of the Bcl-2 family of proteins can block the cell death induced by HDACi [85, 132].

HDACi can activate not only the intrinsic apoptotic pathway but also the extrinsic apoptotic pathway. Nebbioso et al. reported that vorinostat and entinostat induced the expression of TRAIL in AML cells and in AML blast cells isolated from patients. The overexpression observed was the result of the activation of the promoter TNFSF10 [80]. Other studies have demonstrated that HDACi require caspase-8 to exert their cytotoxic effect, also can enhance the extrinsic pathway signal by increasing death receptors (DRs) or ligand expression [82, 133, 134].

We observed a decrease in DNA fragmentation with the caspase-8 inhibitor. In addition, it is well known that activation of caspase-8 can subsequently activate the intrinsic pathway by the cleavage of Bid. We investigated the role of the extrinsic pathway in the synergy of HDACi and adaphostin. Pre-treatment with a pan-caspase inhibitor followed by the synergistic concentrations demonstrated that caspase activation is downstream of changes in the mitochondrial membrane potential (Figure 19). Furthermore, data with I9.2 cells, a caspase-8 deficient cell, demonstrate no significant decreases in DNA fragmentation when compared to their parental Jurkat cells (Figure 17). Together, this data validates that HDACi in combination with adaphostin induce cell death in ALL through the intrinsic apoptotic pathway.

As mentioned before, the mechanism by which the agent combined with HDACi functions also plays a role in how the cell death is induced. The present study used adaphostin that has been shown to induce apoptosis in different hematological

malignancies and solid tumors [112, 117]. Adaphostin is a tyrosine kinase inhibitor originally developed to target BCR/ABL positive cells. Svingen et al. demonstrated that adaphostin activates the apoptotic intrinsic pathway in K562 and blast CML patient samples. Treatment with adaphostin resulted in release of cytochrome c from the mitochondrial, and activation of caspases-9 and -3 [135]. Transfecting cells with a cDNA encoging dominant-negative caspase-9 inhibited the apoptotic changes observed. However, these changes were not inhibited with the transfection of cells with cDNA encoding dominant-negative FADD or with treatment of the blocking antibody anti-Fas [135]. These results demonstrated that adaphostin induce apoptosis via the intrinsic pathway. These observations are consistent with our results demonstrating the involvement of the intrinsic apoptotic pathway in the synergistic cell death induced by adaphostin and HDACi. Other studies support these results not only in BCR/ABL positive cells but also in BCR/ABL negative cells [113].

More detailed studies demonstrated that the main mechanism by which adaphostin induces cell death in different cancer cells is through the production of ROS [113]. These increases in ROS have been shown to be the direct effect of adaphostin binding to complex III of the mitochondrial electron transport chain [114]. In addition, studies have demonstrated that adaphostin has the ability to alter different antioxidant levels like GSH, glutathione S-transferase, superoxide dismutase and heme oxygenase-1 [113, 116, 117]. Consistent with these results, we demonstrated that ALL cells treated with low concentrations of adaphostin alone resulted in a loss of mitochondrial membrane potential starting at eight hours (Figure 18). Increased levels of superoxide and caspase activation at sixteen hours followed this event (Figure 15, 20). Furthermore, the combination of adaphostin with two different HDACi (vorinostat and entinostat) resulted in potentiation of all these events.

Interestingly, several studies have demonstrated that different HDACi have the potential to induce oxidative stress in different cancer cells. These observations directed us to investigate the role of oxidative stress in the synergy between HDACi and adaphostin, which is discussed in the next section.

Induction of oxidative stress by the combination of HDACi and adaphostin.

In order to investigate if oxidative stress plays a role in HDACi and adaphostin synergy, we measured superoxide levels in cells treated with the synergistic combinations. Time course experiments demonstrated that both combinations potentiated superoxide levels starting at sixteen hours of treatment (Figure 20). This data is consistent with observations by others, where adaphostin and different HDACi used as a single agent induce oxidative stress in cancer cells. Ruefli et al. demonstrated that lymphoblastic leukemia cells treated with HDACi resulted in up-regulation of the pro-apoptotic gene Bid. The expression of Bid resulted in disruption of the mitochondria and an increase in ROS levels [87]. Similarly, Rosato et al. demonstrated that HDACi induce ROS in leukemia cells resulting in DNA damage and cell death [75, 108]. Furthermore, several studies combining HDACi with other chemotherapeutic agents have demonstrated a significant increase in oxidative stress in different cancer cells [108-110].

Previous studies have shown that adaphostin depletes GSH levels resulting in accumulation of ROS and subsequent cell death in leukemic cells [113]. Similarly, You et al. demonstrated that the HDACi trichostatin A induced depletion of GSH, which resulted in an increase in superoxide levels and cell death in HeLa cells [136]. More recently, studies combining the agent PEITC, which has been shown to deplete

GSH, with vorinostat also resulted in ROS accumulation and induction of apoptosis in a vorinostat resistant cell line [137].

In order to elucidate if GSH plays an important role in the mechanism by which HDACi and adaphostin induce oxidative stress, we measured the levels of GSH in cells treated with the synergistic concentrations of HDACi and adaphostin. Unlike previous studies, adaphostin alone did not affect GSH levels (Figure 22). This discrepancy can be explained by the concentration used in the previous study, where cells were treated with 10 μM of adaphostin for three hours. In contrast in the present study, the highest concentration used was 750 nM for twenty-four hours. In addition, the previous study used CML cells compared to the present study where we used ALL cells, in which the drug can act by different mechanisms. Similarly to adaphostin, vorinostat alone failed to decrease GSH levels. However, the combination of vorinostat plus adaphostin slightly decreased GSH levels when compared to vorinostat alone. Interestingly, entinostat alone decreased the GSH levels and was further decreased by the combination with adaphostin. However, the decrease in GSH failed to achieve statistical significance in all treatments (Figure 20). This differences between vorinostat and entinostat can be explained by the concentration used, being 0.5 μM for vorinostat and 2.5 μM for entinotat. In addition the specificity of the HDACi can be involved in this differences. Future studies can explore the relation of HDACi treatment concentrations and the inhibition of specific HDAC with the regulation of GSH as antioxidant.

Additional experiments using pre-treatment of GSHee followed by the synergistic combinations of HDACi and adaphostin, demonstrated increases in GSH levels (Figure 23). However, these increases failed to block the mitochondrial

disruption and the DNA fragmentation observed by the treatment of both combinations (Figure 23). Based on these results, we can speculate that GSH is not a major player in the induction of oxidative stress by the combination of HDACi and adaphostin. However, we cannot discard the possibility of GSH depletion working in conjunction with other sources of ROS.

To further investigate the role of oxidative stress in the synergy of HDACi and adaphostin, we used the antioxidant NAC. Pre-treatment with NAC followed by the synergistic combinations of HDACi and adaphostin resulted in a significant inhibition of superoxide levels, demonstrating that NAC is working by blocking ROS (Figure 21 A). In addition, DNA fragmentation was significantly inhibited in cells pre-treated with NAC and followed by the treatment of vorinostat plus adaphostin (Figure 21 C). Interestingly, this inhibition was not observed in cells treated with entinostat plus adaphostin (Figure 21 C). These results were consistent with data assessing caspase-3 activation in cells pre-treated with NAC followed by the drug combination (Figure 21 D). An explanation for these results might be that induction of ROS plays an important role in the combination of vorinostat and adaphostin but not in the combination of entinostat and adaphostin. However, pre-treatment with NAC decreased superoxide levels and reversed the loss of mitochondrial potential in both combinations (Figure 21 A-B). Together, the data generated using NAC, suggest that vorinostat and entinostat in combination with adaphostin might have a different mechanism by which induce oxidative stress mediated-apoptosis.

Another mechanism by which HDACi might induce oxidative stress has been linked to their ability to affect the antioxidant Trx. In this case bladder, prostate and breast cancer cells treated with vorinostat resulted in an up-regulation of thioredoxin binding protein (TBP-2), which has been demonstrated to bind to the antioxidant Trx

and inhibit its activity [92]. Furthermore, Ungerstedt and colleagues demonstrated that multiple myeloma and SV-40 transformed cells treated with vorinostat or entinostat resulted in ROS-dependent apoptosis. However, normal human lung and breast fibroblast cells treated with HDACi resulted in elevated levels of Trx [93]. These elevations in Trx were correlated to HDACi resistance in normal cells. siRNA towards Trx sensitized normal cells to HDACi [93]. More recently in vivo studies demonstrated that vorinostat can change the oxidation state of Trx toward a more oxidized state, which correlated with decreases in enzymatic activity [138]. These studies demonstrated that Trx plays a role in the ROS induction by HDACi.

We tested the possibility of Trx to be involved in the synergy between HDACi and adaphostin. Contrary to previous studies we found that the synergistic combinations did not affect TBP-2 mRNA levels (Appendix). We cannot discard the role of Trx in the combination of HDACi and adaphostin, since this combination can affect Trx using a different mechanism other than via TBP-2. Recent data demonstrate that HDACi induce acetylation of Trx [62]. However, if the acetylation of Trx affects its activity is not known. To test this possibility, we measured Trx acetylation in cells treated with HDACi and adaphostin. Interestingly, in our model, entinostat induced Trx acetylation and this acetylation was further increased with the combination of entinostat and adaphostin. However this level of Trx acetylation was not observed in cells treated with vorinostat and adaphostin (Figure 24). Control samples treated with higher concentrations of vorinostat (5 μM) resulted in acetylation of Trx. This observation demonstrated that one of the differences in Trx acetylation induced by vorinostat plus adaphostin or entinostat plus adaphostin is the concentrations used in each combination. The low concentration of 0.5 μM vorinostat

used in combination with adaphostin did not result in Trx acetylation. In contrast, 2.5 μM entinostat used in combination with adaphostin resulted in increased Trx acetylation. In order to test if Trx acetylation can be linked to its enzymatic inhibition, we measured Trx activity in cells treated with the synergistic combinations. We found that the combination of adaphostin with HDACi did not affect Trx activity (Figure 24). Together this data suggests that Trx is not a major source for the induction of ROS by HDACi and adaphostin combinations. However, these experiments were performed looking at Trx1. Trx has three different isoforms, Trx-1, Trx-2 and Trx-3. Trx-1 and Trx-2 work mainly as antioxidants. Trx-1 works mainly in the cytosol, where as Trx-2 is more directed to the mitochondria [22]. Future studies need to be directed to specifically study Trx-2. This will help to elucidate if Trx-2 plays a role in this combination. Furthermore, additional studies may reveal if different HDACi have better affinity for one of the Trx isoforms, which has not been studied yet.

In order to better elucidate the possible sources of ROS and to understand the differences between both combinations, we performed a gene array examining the expression of different human genes involved in oxidative stress. Results demonstrated a differential regulation of genes induced by the combination of adaphostin-entinostat and adapostin-vorinostat combinations (Table 1). Differences between the combination of vorinostat plus adaphostin and entinostat plus adaphostin were analyzed. Some genes differently affected by one combination versus the second combination include aldehyde oxidase 1, peroxiredoxin-6, metallothionein, angiopoietin-like 7 and glutathione peroxidase-6.

Aldehyde oxidase 1 (AOX1) is one of the genes that can be linked to a possible mechanism by which the combination of entinostat plus adaphostin differs from the combination of vorinostat plus adaphostin. AOX1 is member of the molybdenum hydroxylase family, which also includes xanthine oxireductase [139, 140]. Its major function is to catalize the oxidative hydroxylation of different substrates including: nitrogen-containing heterocyclic compounds, aliphatic and aromatic aldehydes. Its actual physiological substrate is unclear but recent data support its involvement in the metabolism of certain drugs and the detoxification of xenobiotics [139, 140].

In addition to catalize the oxidative hydroxylation, AOX1 also can function as an oxidase transferring electrons to oxygen resulting in the production of superoxide and hydrogen peroxide [139, 140]. Our array data demonstrates that AOX1 is 5.6 fold upregulated in cells treated with the combination of entinostat plus adaphostin. In contrast no changes were observed in cells treated with vorinostat plus adaphostin. Since AOX1 has been associated with the production of superoxide this suggests a possible mechanism by which entinostat plus adaphostin induces superoxide. To the present and to our understanding there is no data showing the effect of NAC in the production of ROS by AOX1. It will be interesting to test the effect of NAC in AOX1. AOX1 can be a possible mechanism in which NAC do not affect its action explaining the differences in inhibition of DNA fragmentation observed only in the combination of vorinostat plus adaphostin and not in entinostat plus adaphostin. Presently there is no a direct correlation between expression of AOX1 and cancer. However as mentioned before, studies has implicate this enzyme with the metabolism of different drugs. Some of these drugs include tamoxifen, methortrexate and 6-mercaptopurine [139]. Studies suggest that AOX1 compete with cytochrome P450, which is a phase I

enzyme involved in the metabolism of different drugs and xenobiotics [140]. These observations support the involvement of AOX1 in the metabolism of drugs.

Recent data published by Maeda and colleagues demonstrated that AOX1 gene is regulated by Nrf2 which binds to the ARE element located in the 5'-upstream region of the AOX1 gene resulting in its expression. This was observed upon the exposure to electrophilic agents [141]. Interestingly, Wang et al. reported Nrf2 activation by HDACi resulting in neuroprotection [142]. It will be interesting to study the relation of HDACi, Nrf2 and AOX1 in the combination of entinostat plus adaphostin.

Efficacy of HDACi and adaphostin *in vivo* studies

Several *in vivo* studies have demonstrated the efficacy of vorinostat in combination with other chemotherapies for the treatment of different types of cancer [143-145]. These studies use different doses and schedules, ranging from 50 mg/kg daily to 200 mg/kg daily. These differences can be explained by the use of different types of cancer xenografts. In addition, the route of administration in these studies vary with intraperitoneal injections being the most commonly used. Using a transgenic mouse model of acute promyelocytic leukemia, He, et al. demonstrated that daily administration for four weeks of 50 mg/kg vorinotat in combination with 1.5 mg/kg retinoic acid, resulted in increased survival compared to single agent administration [143]. In a second study, daily administration of 150 mg/kg of vorinostat significantly inhibited the growth of fribrosarcoma cells in athymic nude mice. Furthermore, this inhibition was dramatically increased by the combination of 2.2 mg/kg of doxorubicin, demonstrating again the efficacy of vorinostat in combination with other chemotherapies [144].

The main objective of the *in vivo* studies performed in this study, was to investigate if the synergy observed between vorinostat and adaphostin can be recapitulated *in vivo*. In general, nude mice have been used as an *in vivo* model for solid tumors. In contrast NOD/SCID mice have been used for hematological malignancies, demonstrating to be more effective in this type of cancer [146]. Using different NOD/SCID models we studied the synergy of vorinostat in combination with adaphostin.

First, we used NOD-SCID-IL-2Rgnull mice, which were injected with 100,000 Molt-4 cells per mice. Directed by previous finding in the literature, we selected the drug dose. Mice were treated thrice weekly with 125 mg/kg of vorinostat [124], 50 mg/kg of adaphostin [125] or the combination of these doses. Results demonstrated that cell engraftment occurred at week four post-inoculation (Figure 26). After one week of drug administration, mice treated with adaphostin died suddenly. Subsequent experiments demonstrated that high doses of adaphostin administrated frequently could induce gastrointestinal (GI) toxicity (Figure 27). Interestingly, mice treated with the combination remained alive for four weeks, suggesting a possible antagonistic effect between the two drugs. After four weeks, we observed a decrease in cell count in mice treated with vorinostat and the combination compared to control mice. However all mice died at the same time due to leukemia. Based on these results we cannot draw any conclusions.

Since we suspected adaphostin toxicity, we decided to decrease the dose to 25 mg/kg of adaphostin and 50 mg/kg of vorinostat. In a second pilot study using NOD/SCID mice, the previously mentioned doses were administrated alone or in combination five days a week. In this occasion we observed GI toxicity in mice treated with adaphostin or the combination after two week of treatment. Decreased doses of

12.5 mg/kg of adaphostin delayed the GI toxicity. In this occasion we started to see GI toxicity again after six weeks of treatments. In term of cells engraftment, we did not observe any engraftment.

For the first time, we report here, GI toxicity as a result of adaphostin treatment administrated at 25 mg/kg daily for two weeks. Limited *in vivo* studies have been reported using adaphostin. Li et al. studied the pharmacokinetics of adaphostin using plasma from different models [125]. These models included mice, rats, dogs and human plasma. They found that adaphostin was most stable in human and murine plasma. In addition, mice were treated with 50 mg/kg of adaphostin using different routes of administration. They concluded that bioavailability following intraperitoneal administration was greater than other administration routes [125]. Based on these observations we selected the adaphostin dose and administration route. However we observed high toxicity using this dose. This discrepancy can be explained by the treatment duration and the mouse strain. The previous study treated CD2F1 mice once at high doses (50 mg/kg) for a few hours [125]; in contrast we treated NOD-SCID mice at 25 mg/kg thrice for a week.

Toxicity tests using different adaphostin doses and administration schedule helped us to elucidate the best dose and schedule resulting in 8 mg/kg of adaphostin administering thrice weekly. This dose was consistent with previous studies administrating adaphostin in a glioblastoma orthotopic model [147]. Finally, this dose was combined with 50 mg/kg of vorinostat, which was administered for five days a week, resulting in no sign of toxicity.

We observed cell engraftment in NOD-SCID-IL-2Rgnull mice after four weeks post-injection. However, NOD/SCID and NOD-Cg-Rag1 mice failed to achieve Molt-4 engraftment. Even though studies have demonstrated that NOD/SCID and mice with

Rag-1 mutations are better for leukemia engraftments, some factors in these mice can affect the engraftment of cancer cells. Hudson and colleagues performed a comparison between Nude, SCID, NOD/SCID and Rag-1 deficient mice [148]. They injected different cells from lymphoid malignancies, including Molt-4. The results demonstrated that NOD/SCID mice had better engraftment compared to the other mouse strains [148]. However, in contrast to our model, they injected 1 x 10^7 Molt-4 cells subcutaneously instead of intravenously. Furthermore, in order to optimize tumor growth, they mixed Molt-4 cells with HT1080 fibrosarcoma cells [148]. While subcutaneous injections allow rapid tumor growth, this model does not provide the tumor microenvironment to accurately model the disease, which can result in false-positive results. In contrast, orthotopic models, where tumor growth arises in the tissue of origin, can mimic the disease with more accurately [149].

Our results demonstrated that NOD-SCID-IL-2Rgnull mice had better cell engraftment compared to NOD/SCID or NOD-Cg-Rag1. Even when NOD/SCID and Rag negative mice lack from the majority of the immune system components, they can still possess low levels of natural killer (NK) cells. In addition, studies have demonstrated that with aging these mice can develop high levels of immunoglobulin, which resulted in decreased engraftment [150]. Furthermore, engraftment of T-lineage ALL cells has lower success rates compared to B-lineage cells in these mice [151]. In contrast to NOD/SCID mice, NOD-SCID-IL-2Rgnull completely lack NK cells, which make this a better model for T-ALL cell engraftment [152].

For future directions, the use of NOD-SCID-IL-2Rgnull mice is recommended. As a second option and in order to improvement the chances of Molt-4 cells engraftment in NOD/SCID mice, Molt-4 can be mixed with HT1080 fibrosarcoma cells.

This last approach has been proven to enhance the cell engraftment [151, 153]. In addition, further immunosuppression can be achieved using irradiation [148].

Conclusion and future directions

In summary, we found that for the first time the ROS generating agent adaphostin synergizes with two different HDACi (vorinostat and entinostat) in ALL cells. Importantly, the synergistic concentration did not affect normal PBMC, demonstrating that this effect is selective for T-ALL cells. This synergy is mainly driven by the activation of the intrinsic apoptotic pathway, where time course analysis showed that perturbation of the mitochondrial membrane potential is the earliest event observed. Following this event, increasing levels of superoxide, caspase activation and ultimately cell death were observed.

Pre-treatment with the antioxidant N-acetylcysteine (NAC) blocked ROS generation and reversed the loss of mitochondrial membrane potential in both combinations. However, apoptosis was only blocked by NAC in cells treated with vorinostat-adaphostin; but not with entinostat-adaphostin, suggesting that these combinations work by different redox mechanisms. Previous adaphostin and/or HDACi targets, like Trx, GSH, were tested to try to elucidate the source of oxidative stress in these combinations. We concluded that Trx and GSH are not the major source of ROS in these combinations. However gene array data, examining the expression of genes involved in oxidative stress, resulted in genes differentially regulated between cells treated with vorinostat-adaphostin and entinostat-adaphostin. These target genes includes: aldehyde oxidase 1, peroxiredoxin-6, metallothionein, angiopoietin-like 7 and glutathione peroxidase-6. From these a promising candidate to follow-up is aldehyde oxidase 1. This gene is involved in the production of superoxide

and drugs metabolism. This was upregulated in the combination of entinostat plus adaphostin but not changes were observed in the combination of vorinostat plus adaphostin.

Future directions include the validation of the different genes, specially AOX1, which were differentially regulated between both combinations. Results from these experiments will help to explore different pathways that can be affected by these combinations. In addition the development of an *in vivo* model is of utmost importance to test the clinical relevance of HDACi plus adaphostin combinations for the treatment of T-ALL. Suggestions will be to employ the initially used NOD-SCID-IL-2Rgnull mouse model with the dose regimens characterized in our toxicity test. In addition, the use of ALL patient samples can be used as a second alternative to test if HDACi synergize with adaphostin.

Understanding the mechanism involved in these combinations will advance scientific knowledge of how the action of HDACi could be augmented in leukemia models. Moreover, this information could be used for the development of effective clinical trials combining HDACi with other anticancer agents.

APPENDIX:

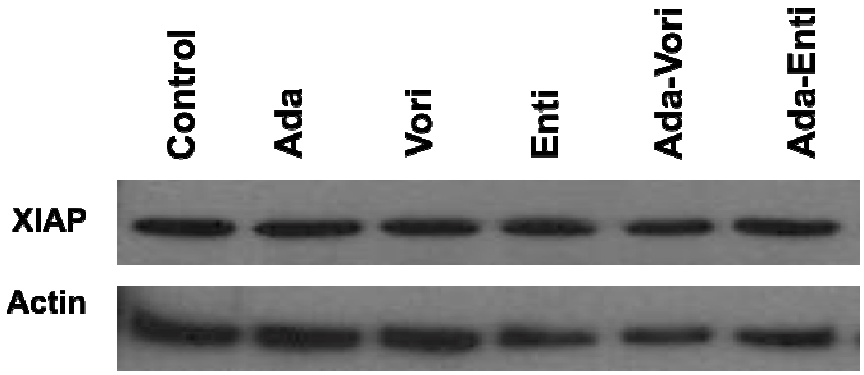


Figure A1. Synergistic combinations do not affect XIAP. 5 x 10^6 Jurkat cells were treated with the synergistic combinations of 0.5 µM Ada and 0.5 µM Vori or 0.75 µM Ada and 2.5 µM Enti or either agent alone. After 24 h of incubation cells were harvested and protein lysates were prepared. XIAP and total actin were measured by Western blot.

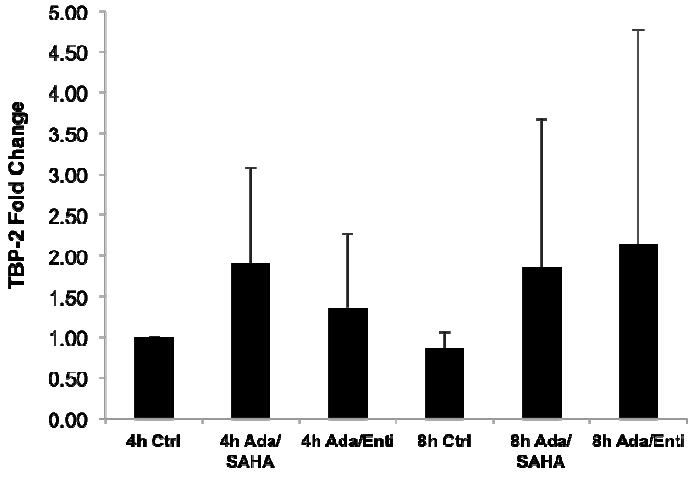


Figure A2. TBP-2 expression in cells treated with the synergistic combinations at 4 h and 8 h. 5 x 10^6 Jurkat cells were treated with the synergistic combinations of 0.5 µM Ada and 0.5 µM Vori or 0.75 µM Ada and 2.5 µM Enti. After 4 h and 8 h cells were harvested and RT-PCR for

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Vita

Nilsa Rivera-Del Valle was born in Bayamon, Puerto Rico on June 14, 1977, the daughter of Carlos R. Rivera and Felicita Del Valle. After completing her high school education at San Pedro Martir Catholic School in 1995, she entered Inter American University of Puerto Rico in Cupey, Puerto Rico. She received the degree of Bachelor in Science with a major in Biology and two minors in Microbiology and Biomedical Sciences in May 2001. She was accepted as a Pre-IRTA Intern at the National Institute of Health (NIH). For the next year she worked in a laboratory of gene expression in the National Institute of Child Health and Human Development (NICHD), in Bethesda, Maryland. After completing her internship at NIH, she returned to Puerto Rico and worked with Elli Lilly Inc. in the area of Quality Control. In August 2003 she entered The University of Texas Health Science Center at Houston Graduate School of Biomedical Sciences. She received the degree of Master in Science in the field of Toxicology in July 2007. In August 2008, she joined Dr. Joya Chandra's laboratory to pursue the degree of Doctor in Philosophy in the field of Experimental Therapeutics. Thus far, her work in Dr. Chandra's laboratory has resulted in two review articles and one research article.