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NAPLES SITE- *Scientific Coordinator Prof. Francesco Salvatore*

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"Evaluation of Histone Deacetylase Inhibitor effects on thyroid cancer"

Supervisor:

Prof. Alfredo Fusco

Internal Supervisor:

Prof. Antonio Simeone

External Supervisor:

Dr. Hinrich Gronemeyer

Ph.D. student:

Dr. Eleonora Borbone

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Abstract

Histone deacetylases (HDACs) play a crucial role in the proper regulation of cellular functions through their connection with chromatin and transcriptional regulation. Alterations in HDAC activity have been reported in several types of cancer encouraging development of HDAC inhibitors (HDACis) for cancer treatment. The antitumor activity of HDACi has been demonstrated, in clinical trials, in both solid and non solid neoplasias at doses well tolerated by patients. However, the molecular basis for their tumor selectivity is unknown. Anaplastic thyroid carcinoma (ATC) is one of the most aggressive malignancies, having a poor prognosis and being refractory to conventional chemo- and radiotherapy. To the aim to find an innovative therapy for the treatment of ATCs, we studied the effects of two potent HDACis, SAHA and MS-275, on rat thyroid cell lines transformed by the *v-ras*-Ki oncogene which is frequently mutated in ATCs. We show that: i) HDAC 1 and HDAC 2 are overexpressed in anaplastic thyroid carcinomas compared to normal thyroid; ii) SAHA and MS-275 induce apoptosis selectively in completely transformed rat thyroid cells; iii) TNF-related apoptosis-inducing ligand (TRAIL) is the main mediator of cell death induced by SAHA; iv) SAHA stabilize TRAIL protein by affecting its proteasome-mediated degradation.

1 INTRODUCTION

1.1 Thyroid gland

The thyroid gland, which is one of the largest endocrine organ in humans, regulates systemic metabolism through thyroid hormones. It is composed of spherical follicles that selectively absorb iodine from the blood for that production of thyroid hormones (T3 and T4). The follicles are surrounded by a single layer of thyroid epithelial cells named follicular cells, which synthesize and secrete T3 (L-triiodothyronine) and T4 (L-thyroxine) hormones under the control of the hypothalamic–pituitary axis with negative feedback by the thyroid hormones (Kondo T, 2006) (Figure 1). Thyrotropin-releasing hormone (TRH), which is secreted from the hypothalamus, stimulates the release of thyroid-stimulating hormone (TSH) from the anterior pituitary gland. TSH stimulates the follicular cells to synthesize and secrete thyroid hormones.. Parafollicular cells (or C cells) are in the interstitial spaces outside the thyroid follicles and produce the calcium-regulating hormone calcitonin.

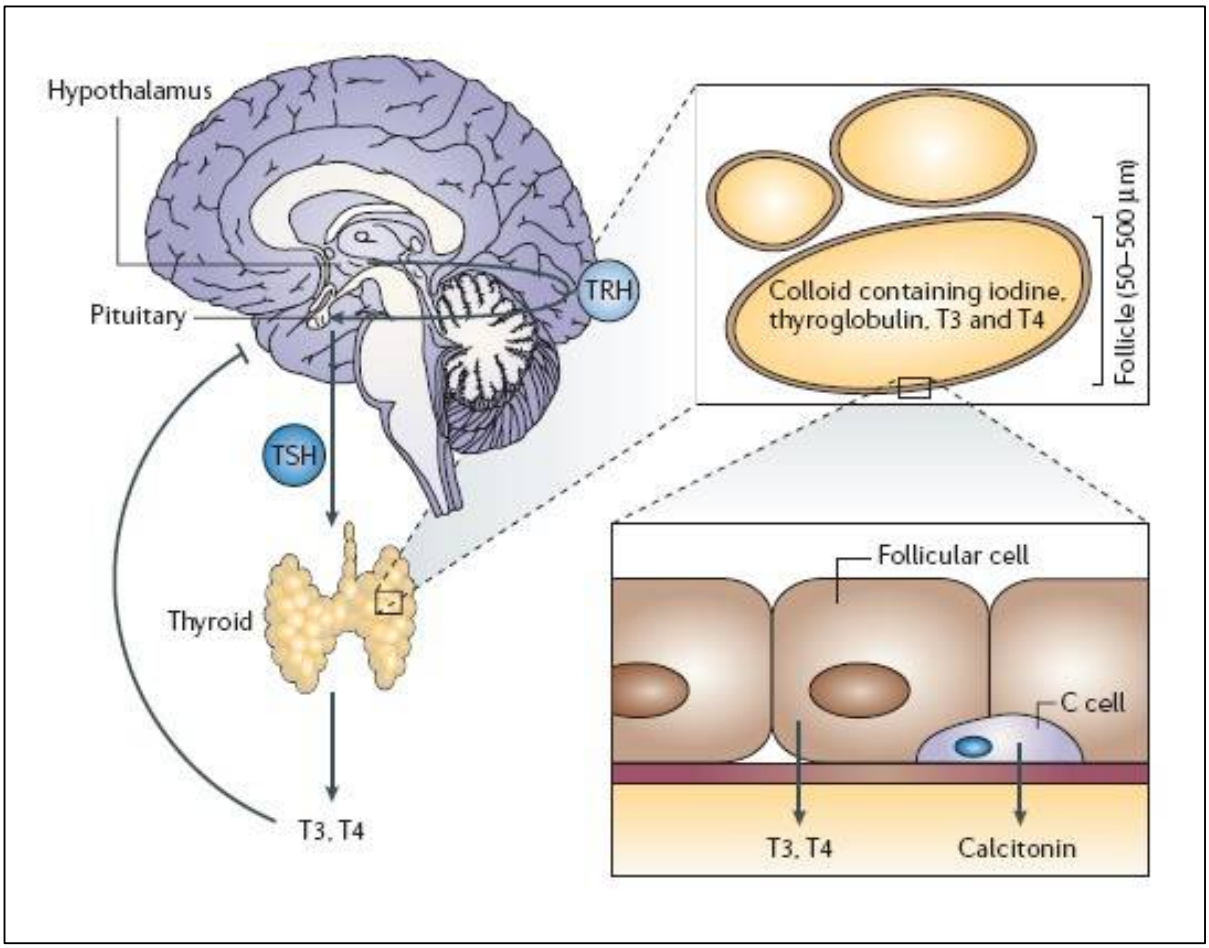


Figure 1. Schematic representation of thyroid gland structure and function. Thyroid follicular cells synthesize, store and secrete thyroid hormones T3 and T4 under the control of the hypothalamic-pituitary axis with negative feedback by the thyroid hormones.

1.2 Thyroid cancer

Tumors are the result of the accumulation of different modifications in critical genes involved in the control of cell proliferation. In a large number of carcinomas with worst prognosis, lesions are not diagnosed until the disease is at an advanced stage. Although

various therapeutic approaches are followed in clinical practice, most of them are not life-saving.

Thyroid nodules can be hyperplastic benign adenomas or malignant lesions, and can be derive from thyroid follicular epithelial cells or C cells. However, more than 95% of thyroid carcinomas are derived from follicular cells (Kondo T, 2006) and are the most common endocrine malignancies, with an estimated 25,000 new cases diagnosed annually in the United States. Conversely, only 3% of thyroid tumors, referred to as medullary thyroid carcinoma, are of C-cell origin.

Thyroid neoplasms represent a good model for studying the events involved in epithelial cell multistep carcinogenesis, because they comprise a broad spectrum of lesions with different degrees of malignancy which are diagnosed on the basis of histological and clinical parameters. Follicular cell-derived thyroid tumors include: 1) benign adenomas, which are not invasive and very well differentiated; 2) carcinomas, which are divided into well-differentiated, poorly differentiated and undifferentiated types. Well-differentiated thyroid carcinomas are papillary (PTC) and follicular (FTC) types, being differentiated and having a good prognosis (Kondo T, 2006; Saltman B, 2006). Most well-differentiated thyroid carcinomas are 2–4 times more frequent in females than in males and manifest in patients who are between years of age. The papillary

carcinoma is defined as a malignant epithelial tumor, showing papillar and follicular architecture and typical nuclear alterations (Hedinger C,1989). Particularly, “occult” papillary carcinomas are slow-growing and clinically silent carcinomas. PTCs represent more than 70% of thyroid malignant tumors and occurr two to three-fold more frequently in females (Salabe GB, 1994). Exposure to ionizing radiation and radioactive contamination of the environment increase the risk of developing this kind of cancer as evidenced by the effects of the atomic bombs of Hiroshima and Nagasaki (1945), nuclear testing in the Marshall Islands (1954) and Nevada (1951–1962), and the more recent nuclear accident in Chernobyl (1986) (Nikiforov Y, 1994; Kazakov VS,1992). After the Chernobyl disaster, the effects of radiation exposure were most pronounced in children. External beam-radiation exposure in childhood for the treatment of benign conditions of the head and neck also increased the risk of papillary carcinoma (Ron E, 1995).

Differently from papillary carcinomas, which can invade local lymph nodes, follicular carcinomas are characterized by haematogenous spread and distant metastasis. FTCs represent 10% of thyroid carcinomas, they are more common in females than in males and rarely associated with radiation exposure.

Poorly differentiated (PDTC) and undifferentiated anaplastic carcinomas (ATC) seem to derive from the progression of

differentiated carcinomas (Van der Laan BF, 1993). Although the ATC represents 2-5% of thyroid malignant tumors, it is one of the most lethal human neoplasms being rapidly–growing, very aggressive and always fatal. Anaplastic cancers invade adjacent structures and metastasize extensively to cervical lymph nodes and distant organs such as lungs and bones. Finally, PDTCs, representing 7% of thyroid carcinomas, are morphologically and behaviourally intermediate between well-differentiated and undifferentiated thyroid carcinomas. Medullary carcinomas of the thyroid are significantly less common than follicular cell-derived thyroid tumor and include multiple endocrine neoplasia 2A and 2B (MEN2A, MEN2B) and familial medullary thyroid carcinoma. This type of thyroid tumor is characterized by gain-of-function mutations of RET proto-oncogene.

1.3 Genetic lesions in thyroid carcinomas

The involvement of several oncogenes has been demonstrated in thyroid carcinomas (Figure 2). Mutations or rearrangements in genes encoding RET, NTRK1, BRAF or Ras are detectable in nearly 70% of all cases in well-differentiated thyroid carcinoma (Table 1) (Kondo T, 2006). RET is a transmembrane receptor-tyrosine kinase endcoded by RET proto-oncogene which is rearranged in sporadic

and radiation-associated papillary carcinoma. The most common RET chimeric genes are H4(CCDC6)–RET (also known as RET/PTC1) and ELE1–RET (also known as RET/PTC3) in papillary carcinoma (Kondo T, 2006). The neurotrophic receptor-tyrosine kinase NTRK1 (also known as TRK and TRKA) is another proto-oncogene encoding a transmembrane tyrosine-kinase receptor for nerve growth rearranged in 5-13% of sporadic but only 3% of radiation-induced papillary thyroid tumors. TPM3, TPR and TFG are the main fusion partners in the chimeric oncogenes TRK, TRK-T1 and TRK-T2, and TRK-T3 (Kondo T, 2006). BRAF is a serine/threonine kinase involved in cell proliferation pathways. BRAFV600E is the most common alteration in sporadic papillary carcinoma. BRAF mutations are found in 29–69% of papillary thyroid carcinoma, in up to 13% of poorly differentiated thyroid carcinoma and 35% of undifferentiated anaplastic thyroid carcinoma but not in follicular thyroid carcinoma (Kondo T, 2006).

Genetic alteration	PTC	FTC	PDTC	ATC
RET rearrangement	13-43%	0%	0-13%	0%
Braf mutation	26-69%	0%	0-13%	10-35%
NTRK1 rearrangement	5-13%	Unknown	Unknown	Unknown
Ras mutation	0-21%	40-53%	18-27%	20-60%
TP53 mutation	0-5%	0-9%	17-38%	67-88%

Table1. Genetic defects in papillary (PTC), follicular (FTC), poorly differentiated (PDTC) and anaplastic (ATC) thyroid carcinomas.

Ras mutated is one of the most common genetic lesions found in human tumors. Ras proto-oncogenes known as HRAS, KRAS and NRAS play a crucial role in thyroid tumorigenesis. A low incidence of Ras mutation was found in well-differentiated thyroid carcinoma whereas this phenomenon is more frequent in poorly differentiated and undifferentiated thyroid carcinoma, thus indicating its importance in thyroid tumor progression.

The critical role of *ras* gene activation in some thyroid cancer histotypes was confirmed by the induction of thyroid follicular carcinomas associated to lung metastasis following the injection of the Kirsten murine sarcoma virus into the thyroid gland of adult Fischer rats (Portella G, 1989). The important role of *ras* oncogene in thyroid cancer was also demonstrated in human follicular and anaplastic carcinoma where the frequency of mutation in this gene is about 50% (Nikiforova MN, 2003). Finally, PAX8-PPAR-γ rearrangements which juxtaposes the thyroid transcription factor PAX8 to the peroxisome proliferator-activa ed receptor (PPAR-γ) and impairment of the TP53 tumor suppressor gene were also reported in follicular and anaplastic carcinomas, respectively (Suarez HG, 1990; Fagin JA,1993)

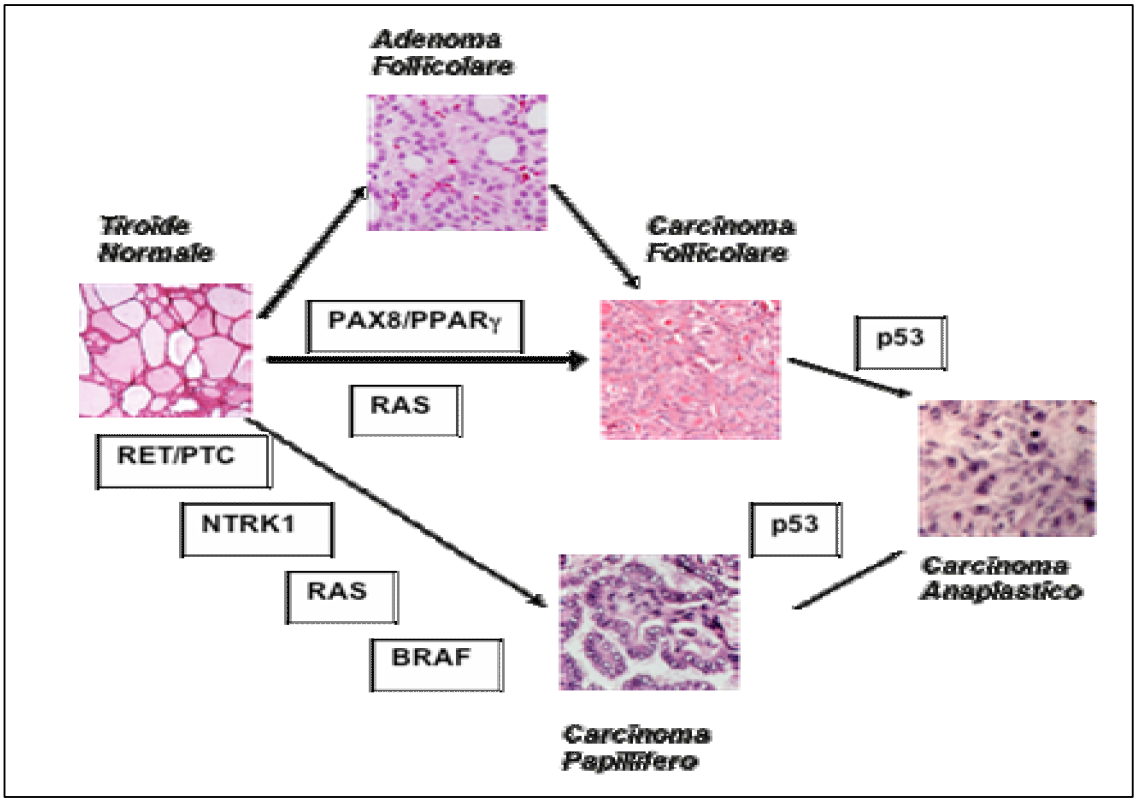


Figure 2. Schematic representation of genetic alterations involved in neoplastic transformation in different histotypes of thyroid carcinomas.

1.4 Therapy

Surgical resection and radioactive iodine can be an effective treatment for well-differentiated tumors. In fact, most thyroid cancer cells retain the ability to absorb and concentrate iodine. This provides a perfect "chemotherapy" strategy. Radioactive Iodine is given to the patient with thyroid cancer after their cancer has been removed. Remaining cancerous thyroid cells will absorb iodine and will be destroyed by the radioactivity (Kondo T, 2006). However, undifferentiated anaplastic thyroid carcinomas have a poor prognosis being refractory to conventional chemotherapy and

radiotherapy (Kondo T, 2006). Most of these cancers are so aggressively attached to vital neck structures that they are inoperable and death usually occurs within 1 year of diagnosis. A novel approach that is currently being tested for the treatment of thyroid cancer is the use of epigenetic drugs like Histone Deacetylases Inhibitors (HDACis).

1.5 Epigenetic control of gene expression

In eukaryotes DNA is packaged into chromatin fibers to achieve compaction, enabling the entire genome to fit into the nucleus, and allowing DNA transcription, replication, and repair, when necessary (Figure 3).

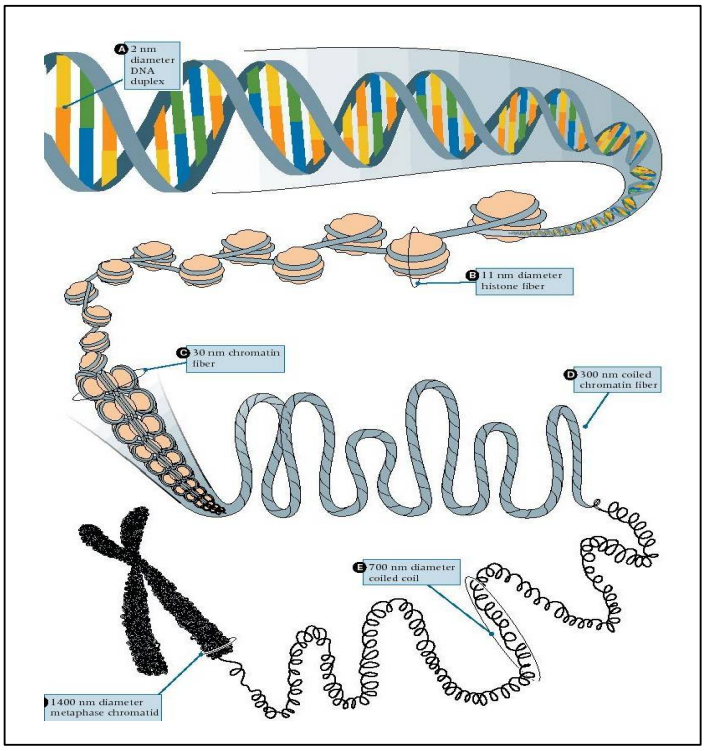


Figure 3. Organization of DNA into chromatin, Nucleosome fibers are packaged into higher order structures giving rise to chromosomes.

The nucleosome is the basic repeating unit of chromatin polymer and consists of 146 bp of DNA wrapped around a histone octamer (Marks PA, 2001). Histones are small basic proteins conserved throughout evolution and consist of a globular domain flanked by a carboxy-terminal and a lysine rich amino-terminal tail composed of positively charged residues. The core histone octamer is formed by two copies of each of histones H2A, H2B, H3 and H4 organized in an H3-H4 tetramer and two H2A-H2B dimers. The binding of histone H1 to linker DNA sequences between nucleosomes leads to a further chromatin compaction, by mechanisms that remain poorly defined.

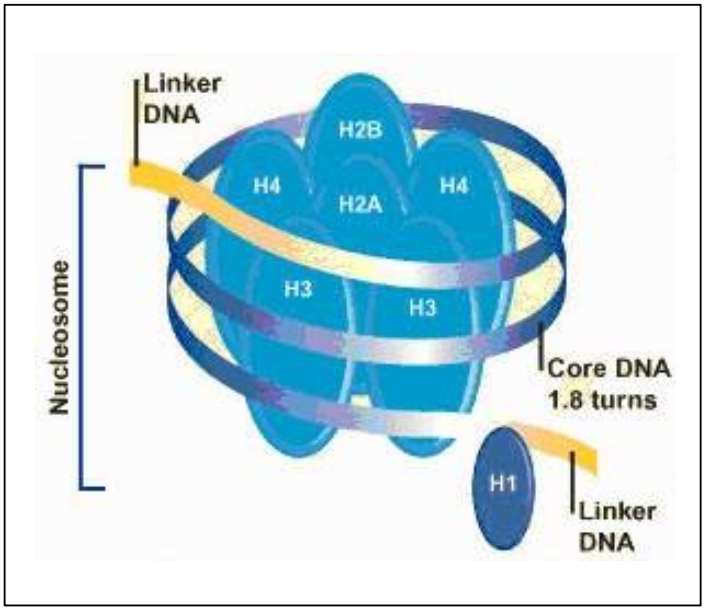


Figure 4. Schematic representation of a nucleosome. DNA is wrapped around core histone octamer which contains two H3-H4 tetramers centrally located and two flanking H2A-H2B dimers. Histone H1 links nucleosomes into higher order structures.

Histone lysine tails protruding from the nucleosome are sites of post-translational modifications including acetylation, methylation and ubiquitination of lysine (K) residues, phosphorylation of serine (S) and threonine (T) residues, methylation of arginine (R) and ADP-ribosylation of glutamic acid residues (Fischle W, 2003; Zhang Y, 2001) (Figure 5).

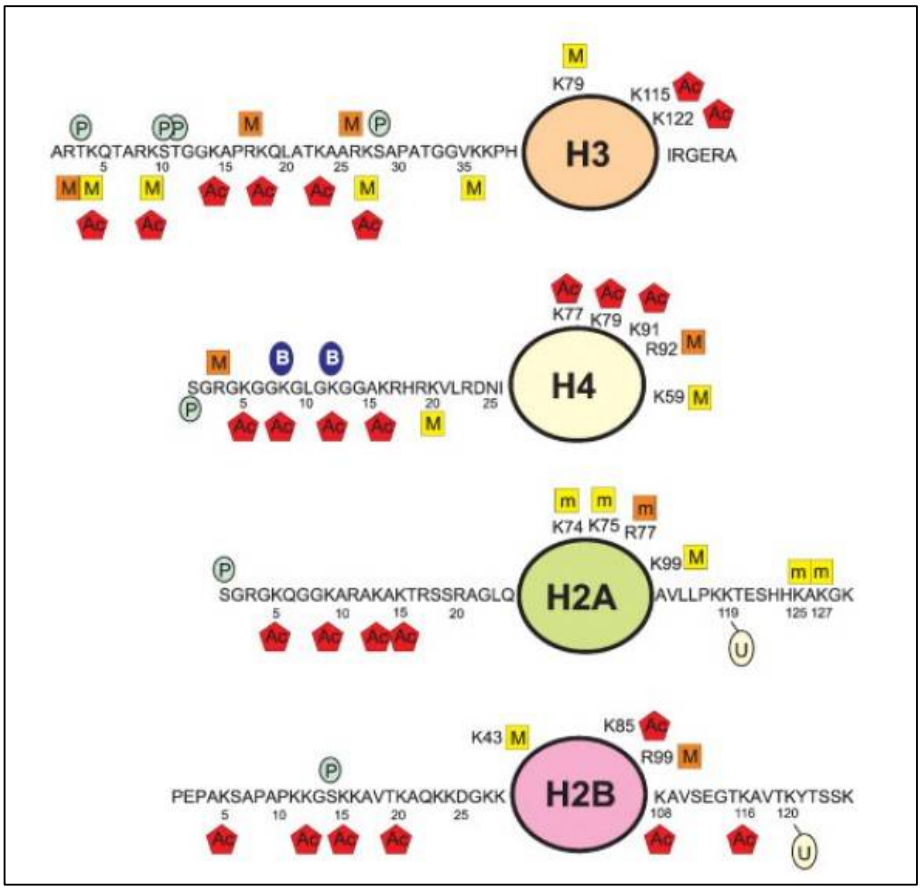


Figure 5. Covalent modifications of the N-terminal tail of the core histones. Acetylations are shown as red hexagons; methylation as yellow and orange squares; phosphorylations as green circles.

Strahl and Allis (Strahl BD, 2000) proposed that distinct histone modifications, on one or more tails, act sequentially or in combination to form a 'histone code' that is, read by other proteins leading to distinct downstream events. This theory states that post-translational modifications can act through two mechanisms that are not mutually exclusive: (i) by structurally changing the chromatin fiber through internucleosomal contacts thus regulating the access of transcription factors to the DNA; and (ii) by generating docking sites for effector molecules that, in turn, initiate distinct biological processes. The histone code is part of the epigenetic information found into the cells. The term “epigenetics” refers to mitotically and meiotically heritable changes in gene expression that are not coded in the DNA sequence itself (Egger G, 2004). Indeed, DNA methylation is another well studied epigenetic mechanism. Methylation at the C-5 position of cytosine residues present in CpG dinucleotides by DNA methyltransferases (DNMTs) is generally considered to facilitate static long-term gene silencing (Lund AH, 2004).

1.6 Histone acetylation

To date, acetylation is the most extensively studied histone post-translational modification and involves the transferring of an acetyl

group from the metabolic intermediary acetyl coenzyme A to the ε-amino group of a lysine residue. The primary effect produced by acetylation is the partial neutralization of the positive charge of the histones, thus decreasing their affinity for the DNA, altering nucleosome-nucleosome interactions and enabling chromatin decondensation to allow transcriptional activation (Jenuwein T, 2001; Vaquero A, 2003). Although it is known that the four core histones (H2A, H2B, H3, and H4) can be acetylated in their N-terminal tails, the H3 and H4 modifications are mainly responsible for the effect on transcription.

The balance between acetylated and deacetylated states of chromatin is regulated by histone acetyltransferase (HAT) and histone deacetylase (HDAC) enzymes (Marks PA, 2001; Minucci S, 2006). HATs transfer acetyl groups to amino-terminal lysine residues in histones, which results in local expansion of chromatin and increased accessibility of transcription factors to DNA, whereas HDACs catalyse the removal of acetyl groups, leading to chromatin condensation and transcriptional repression (Figure 6).

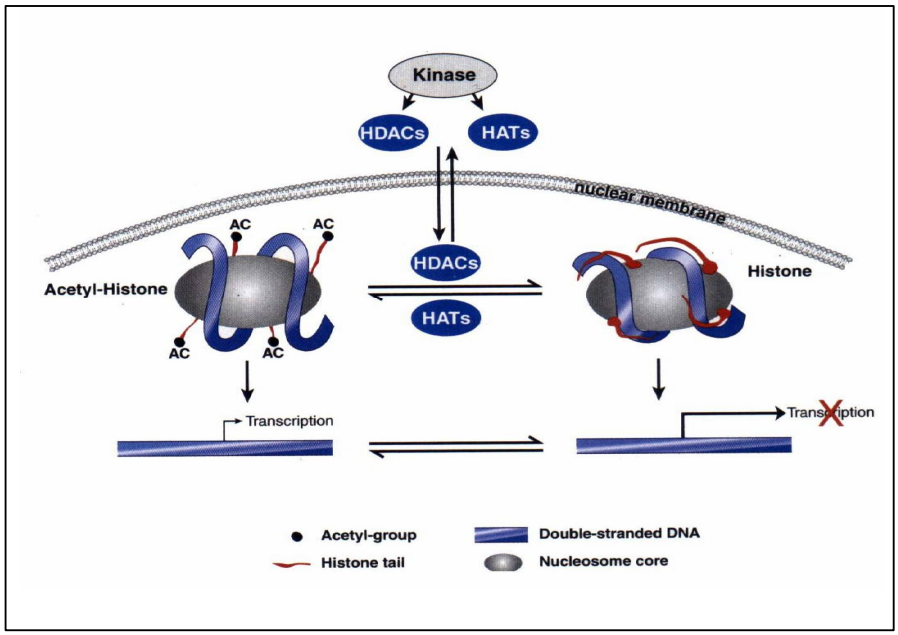


Figure 6. Schematic representation of the equilibrium between HDAC and HAT activities.

1.7 HDAC classification

Mammalian HDACs are divided into four classes on the basis of their sequence homology to yeast HDACs: a) class I includes HDAC 1, 2, 3 and 8 which are homologous to yeast HDAC Rpd3; b) class II is further subdivided into two subclasses, IIa (HDAC 4, 5, 7, 9) and IIb (HDAC 6 and 10), based on their sequence homology and domain organization; class II HDACs are homologous to yeast Hda1; c) class III HDACs are the sirtuins (SIRT1-7) which are homologous to yeast Sir2 family of proteins; d) HDAC11 is phylogenetically different from both class I and class II enzymes and is considered as a separate class named class IV (Gallinari P, 2007)

(Figure 7). HDACs belonging to classes I, II and IV require a zinc ion in the catalytic domain to mediate deacetylation catalysis. Conversely, sirtuins are dependent on NAD^+ as a cofactor (Altucci L, 2009; Minucci S, 2006). Class I HDACs are generally localized to the nucleus, and are ubiquitously expressed in many human tissues (Gallinari P, 2007; Vaquero A, 2003). Class I HDACs have also been shown to interact with factors involved in other functions, such as DNA or histone methyltransferases (Dnmts and Polycomb group proteins) (Vaquero A, 2003). Class IIa HDACs (subtypes 4,5,7 and 9) contain a highly conserved C-terminal deacetylase catalytic domain while Class IIb HDACs (subtypes 6 and 10) are characterized by an additional deacetylase domain, although this duplication is partial in the case of HDAC 10. Class II HDACs can shuttle between the nucleus and cytoplasm, suggesting potential extranuclear functions by regulating the acetylation status of nonhistone substrates (McKinsey TA, 2000). In fact, the second deacetylase domain of HDAC6 has been reported to deacetylate α-tubulin and Hsp90 (Gallinari P, 2007). Class II members participate in skeletal muscle differentiation by interaction with members of the muscle-specific MEF2 transcription factor family (McKinsey TA, 2000; De Ruijter AJ, 2003). HDACs are regulated by gene expression, subcellular localization, and post-translational modifications such as phosphorylation, sumoylation, proteolysis, and

availability of metabolic cofactors (Dokmanovic M, 2005). HDACs, like HATs,, do not bind to DNA directly, but are recruited to multi-protein complexes associated with DNA which differ in their subunit composition. Transcription factors recruit coactivators with HAT activity (e.g. p300/CBP) to regulatory DNA sites, while transcriptional repressors recruit corepressors with HDAC activity (Dokmanovic M, 2005). Class I and class II HDACs are often found as components of larger transcription factor protein complexes: HDAC1 and 2 are frequently found in complex with the histone binding proteins RbAp46/48 (retinoblastoma-associated proteins) and SAP30 (Sin3-associated protein), Sin3 (general transcriptional co-repressor) or MBD3 (methyl-CpG binding domain protein 3), MTA2 (metastasis associated 1), Mi2 (dermatomyositis-specific autoantigen) forming the Sin3 and NuRD (nucleosome remodeling and deacetylase) corepressor complexes, respectively. The components of Sin3 complex impart specificity for its localization to certain regions of the genome and regulate its activity. NuRD complex represses transcription more globally but also interacts with gene-specific transcription factors. The RbAp proteins, present in both complexes, have a role in stabilizing the interaction of each complex with the core histones of the nucleosomes. HDAC3 is found in complex with the nuclear hormone corepressors NCoR (nuclear receptor corepressor) and SMRT (silencing mediator for retinoid and

thyroid hormone receptors). In fact, HDAC3 is especially involved in the repression of genes connected with nuclear receptor signaling. HDAC-containing complexes can interact with a wide variety of transcriptional regulatory proteins, most of them DNA binding proteins, such as the repressors and transcription factors. Class II HDACs also interact with the NCoR, SMRT, and the Bcl6 gene-specific co-repressor BCoR.

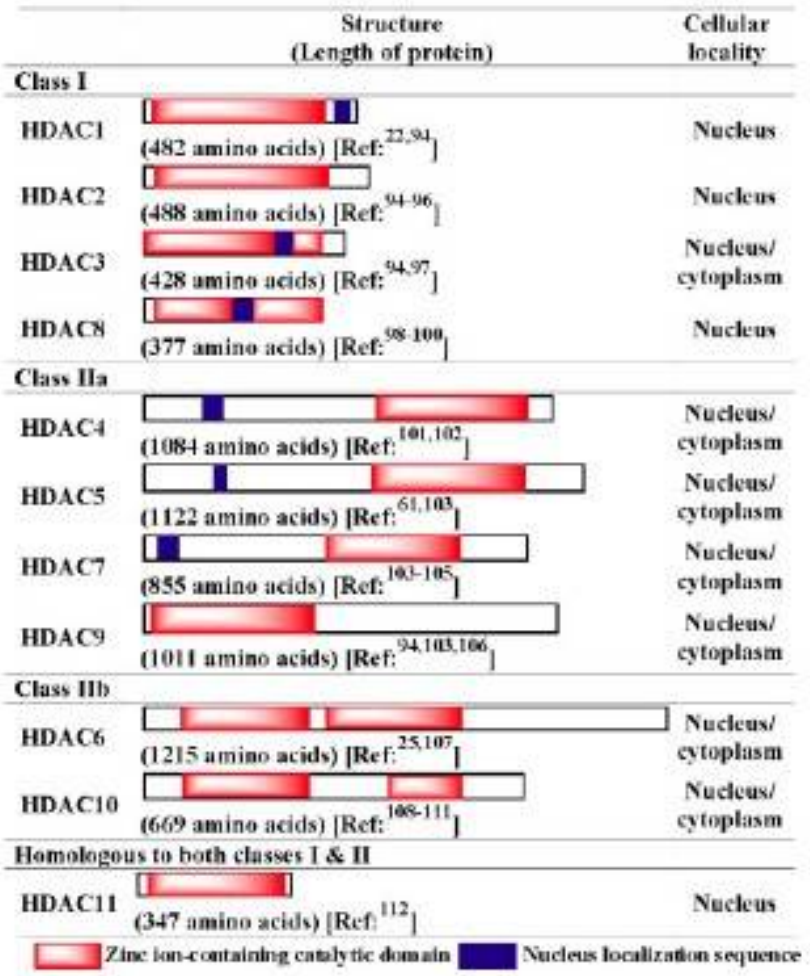


Figure 7. HDAC classification**.** Structure and localization of Class I and II HDAC enzymes.

1.8 Epigenetics and cancer

Cancer is usually characterized by genetic defects such as gene mutations and chromosomal abnormalities, which cause loss of function of tumor-suppressor genes and/or gain of function or hyperactivation of oncogenes. However, there is growing evidence that epigenetic control of gene expression is also crucial to the onset and progression of cancer. Notably, in contrast to genetic modifications, epigenetic alterations are transient and can be reversed, at least partially, by treatment with epigenetic drugs (Bolden JE, 2006). Disruption of HAT or HDAC activity can be associated with the development of cancer (Marks PA, 2001). Translocations, amplifications, deletions and point mutations have been reported for HAT genes (p300, PCAF and CBP) in various human cancers, whereas mutations in HDAC genes are rare in human tumors. In fact, abnormal HDAC activity, due to its overexpression or aberrant recruitment by altered protein partners, is more common in different types of cancer (Marks PA, 2001; Dokmanovic M, 2005; Minucci S, 2006; Bolden JE, 2006). Increased expression of HDAC1 has been detected in gastric cancers, oesophageal squamous cell carcinoma, and hormone refractory prostate cancer, while HDAC2 overexpression has been detected in colon cancer (Dokmanovic M, 2005).

Acute promyelocytic leukaemia (APL) was the first model disease in which the involvement of HDACs was demonstrated (Minucci S, 2006). This form of leukaemia is characterized by an arrest of the leukaemic cells at the promyelocytic stage of myeloid differentiation related to the presence of the fusion protein of retinoic acid receptor-α (RAR) with one of the following proteins: promyelocytic leukaemia (PML) in > 95% of cases, promyelocytic leukaemia zinc finger (PLZF) in 5% of cases or other genes sporadically. RAR functions as a transcription factor. In the absence of retinoic acid (RA), RAR is found at DNA response elements of RA-regulated genes and is associated with HDAC containing complexes; this contributes to the transcriptional silencing of these genes. At physiological concentrations of RA, a conformational switch leads to the release of the HDAC-containing complexes and to the association of RAR with transcriptional co-activators (including HATs) and subsequent transcriptional activation (Minucci S, 2006). PML-RARα and PLZF-RARα recruit HDACs through NCoR and SMRT and cause aberrant transcriptional repression that prevents differentiation (De Ruijter AJ, 2003). Pharmacological doses of retinoic acid induce clinical remission in APL patients expressing PML-RARα. This effect is due to removal of HDAC-dependent repression from RAR target genes and the resulting re-differentiation of the leukaemic cells (Minucci S,

2006). Conversely, patients expressing PLZF–RAR α, which is insensitive to pharmacological doses of RA, require a combined treatment retinoic acid plus HDAC inhibitor to allow HDAC release and reactivation of RAR-dependent gene transcription. The transcriptional repressor LAZ3/BCL6 (lymphoma-associated zinc finger-3/B cell lymphoma 6) is overexpressed in non-Hodgkin's lymphoma, and causes aberrant transcriptional repression through recruitment of HDACs leading to oncogenic transformation (Marks PA, 2001). AML1-ETO is another fusion protein which abnormally recruits HDAC-containing complexes (Marks PA, 2001; Altucci L, 2005). The fusion of the DNA binding domain of the transcription factor AML1 to the entire coding sequence of another transcription factor named ETO, is produced by a translocation common in acute myeloid leukaemia (Altucci L, 2005). However, these fusion proteins promote DNA methyltransferases and histone methyltransferases recruitment leading to compaction of chromatin. Furthermore, deregulation of transcription factors or chromatin modifiers targeting HDACs to specific genomic regions have been observed in solid tumors affecting the balance of histone acetylation. Alteration in histone acetylation status can lead to changes in chromatin structure and transcriptional dysregulation of genes that are involved in the control of cell-cycle progression, differentiation and/or apoptosis. DNA and histone methylation, tightly linked to chromatin

repressive states, also have an important role in tumorigenesis and are dependent on the concomitant action of HDACs (Egger G, 2004; Feinberg AP, 2004; Minucci S, 2006). In these examples, transcriptional repression is mainly mediated by the recruitment of HDACs and provides a mechanistic rationale for the treatment of different types of tumors with inhibitors of HDAC activity.

1.9 Histone deacetylase inhibitors

HDAC inhibitors are divided into at least five distinct classes on the basis of their chemical structure and pharmacological properties such as potency, efficacy, stability and toxicity. The classes are as follows: short-chain fatty acids, hydroxamic acids, electrophilic ketones, cyclic tetrapeptides and benzamides (Figure 8).

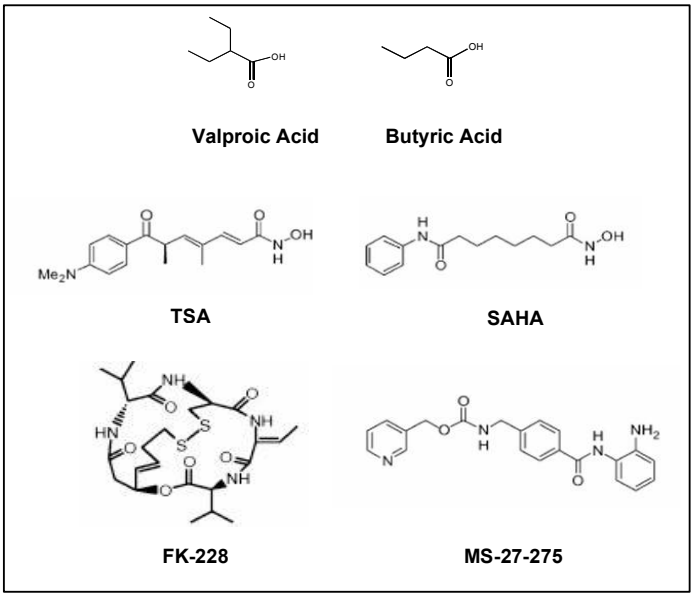


Figure 8. Representative structures of the main members of HDACi classes.

Short chain fatty acids include valproic acid and butyrate which are already used in treating epilepsy, but also inhibit class I HDACs (Glickman MH, 2002). Particularly, valproic acid inhibits HDAC 1 and 2 by decreasing catalytic activity and proteasomal degradation, respectively (Krämer OH, 2003; Yoo CB, 2006). Phase I and phase II clinical trials are ongoing for leukaemias and myelodysplasia to evaluate this drug as an antitumor agent (Dokmanovic M, 2005; Minucci S, 2006). Butyrate was the first HDAC inhibitor to be identified and the related compound phenylbutyrate has been employed in experimental cancer therapy (Marks PA, 2001). However, valproic acid and butyrate are less potent than other HDAC inhibitors and have a short plasma half-life thus requiring high concentration to inhibit HDACs: micromolar and millmolar concentrations *in vitro* and *in vivo*, respectively (Marks PA, 2001; Johnstone RW, 2002; Hellebrekers DM, 2007). In fact, these compounds are unstable and possess an acyl group which cannot make significant contact with the catalytic pocket due to their very short side chain. The structural requirements for a perfect interaction beteen HDACis and target enzyme are shown in Figure9.

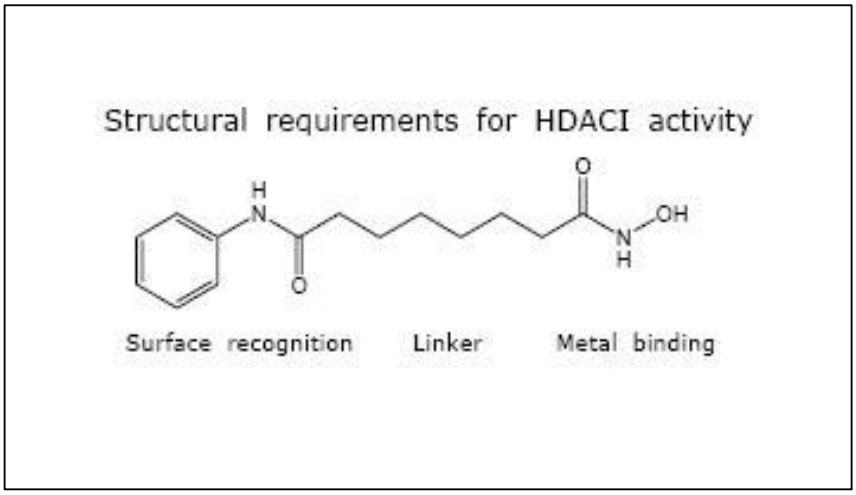


Figure 9. HDACi essential features responsible for optimal interactions with HDAC target

Hydroxamic acids are potent HDACis and consists of a functional group interacting with the HDAC zinc atom, a 5-6 carbon aliphatic chain which mimics a lysine side chain, and a hydrophobic cap moiety which interacts with the edge of the catalytic pocket and could play a role in HDAC selectivity (Johnstone RW, 2003). This class includes trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA), pyroxamide, m-carboxycinnamic acid bishydroxamide (CBHA), oxamflatin, scriptaid. All hydroxamates have the property to inhibit class I and II HDACs at nanomolar concentration *in vitro*, but TSA and SAHA are the most potent and effective inhibitors of this class (Kim DH, 2003; Dokmanovic M, Marks PA, 2005; Marks PA, 2007; Pan L.N., 2007). Moreover, crystallographic analysis showed that both TSA and SAHA interact directly with the catalytic site of

HDAC inhibiting its enzymatic activity (Finnin MS, 1999) (Figure 10). TSA is a fermentation product of *Streptomyces hygroscopicus* used as an anti-fungal agent but it was also the first natural product hydroxyamate discovered to inhibit HDACs (Yoshida M,1995). SAHA has a chemical structure similar to TSA, but was generated from chemical synthesis and shows a weaker inhibitory activity for HDAC than TSA. However, SAHA is effective for the inhibition of tumor growth, both *in vitro* and *in vivo*, at the micromolar concentration, with minimal side effects and has a longer half-life with respect to TSA (Glick RD, 1999; Butler LM, 2000; Kim DH, 2003; Piekarz R, 2004). Among the hydroxamic acids, SAHA has been shown to be the most effective HDACi, capable of inducing early changes in gene expression profile, which include suppression of genes mediating proliferation and survival.

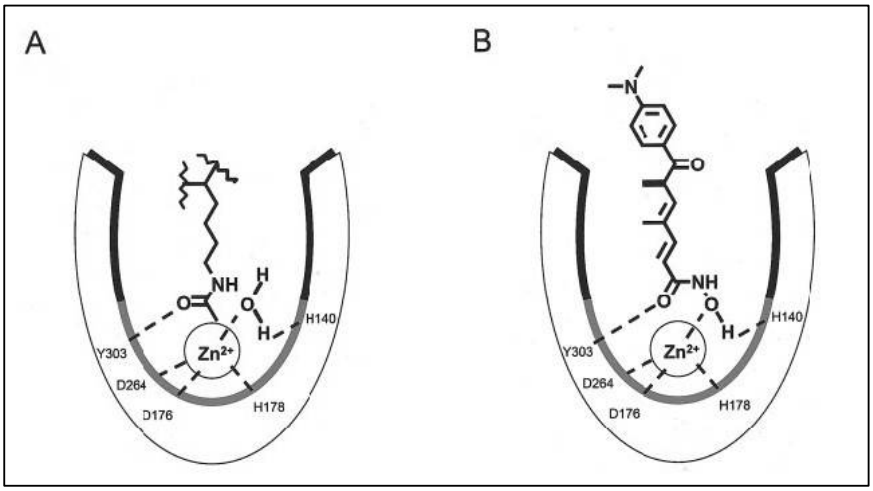


Figure 10. Schematic representation of HDAC catalytic site. (A) Interaction of an acetyl-lysine residue and a water molecule with the zinc cation. (B) TSA chelating Zn^{2+} in the catalytic pocket. Thick black lines represent hydrophobic surfaces. Thick gray lines indicate areas rich in charged aminoacids (aspartic acid and histidine).

Electrophilic ketones, like trifluoromethyl and heterocyclic ketones , are modest HDACis with short half-life and weak antiproliferative activity against tumor cell lines (Gallnari P, 2007).

Cyclic tetrapeptides include depsipeptide (FK-228), apicidin, trapoxin and depudesin which preferentially inhibit class I HDACs (Bolden JE,2006). Depsipeptide is a bacterial metabolite produced as a prodrug which undergoes metabolic activation in the cell through the reduction of the intramolecular disulfide bond by reducing agents (e.g. glutathione). This reduction yields two dulfhydryl groups, one of which is accessible to the catalytic Zn^{2+} of HDACs (Kim DH, 2003; Dokmanovic M, 2005; Gallinari P, 2006; Glaser KB, 2007; Pan LN, 2007). Depsipeptide possesses stronger inhibitor activity against HDAC 1 and 2 than against class II HDAC4 and HDAC6 at nanomolar concentrations (Furumai R, 2002; Bolden JE, 2006). Apicidin is a fungal metabolite able to inhibit HDACs at nanomolar concentrations (Dokmanovic M, 2005). Trapoxin and depudesin are the only HDACis which inhibit the enzyme irreversibly via covalent binding to the catalytic site (Marks PA, 2001). The final class comprises benzamide compounds such as CI-994 and MS-27-275 (MS-275) which inhibit HDAC at micromolar concentration. CI-994 (N-acetyl dinaline) is a relatively weak HDAC inhibitor. MS-275 is a selective inhibitor for HDAC 1, 2 less active against HDAC3 (Hu E, 2003; Nebbioso A, 2005; Bolden JE, 2006; Glaser KB, 2007; Pan

LN, 2007).

1.10 HDACi effects: from *in vitro* models to clinical experimentation

HDACis induce growth arrest, differentiation and cell-death of different hematologic and solid tumor cells in culture (Drummond DC, 2005; Kelly WK, 2005). Many HDACis including TSA, SAHA, MS-275 and FK-228 administered intravenously or intraperitoneally inhibit tumor growth in nude mice transplanted with human solid and nonsolid tumors (xenograft models) such as human breast, prostate, lung and stomach cancers, neuroblastoma, medulloblastoma, multiple myeloma and leukemias with little toxicity (Butler LM, 2000; Marks PA, 2001; De Ruijter AJ, 2003; Marks PA, 2004; Piekarz R, 2004; Drummond DC, 2005). The marker mainly used to monitor dosing and HDAC biological activity in both animal models and cancer patients is the accumulation of acetylated histones in tumor and normal tissues (spleen, liver and peripheral mononuclear cells) (Marks PA, 2001).

Positives results obtained with HDACis in preclinical studies encouraged the introduction of these drugs in clinical trials. Phenylbutyrate, like other members of the short-chain fatty acid class, has a short half-life and inhibits HDACs at high concentration,

thus requiring prolonged intravenous or oral administrations to achieve constant serum concentrations. However, the continuous infusion of the drug has increased the risk of toxic side-effects inducing confusion, somnolence, nausea, vomiting, dyspepsia and fatigue in treated patients (Marks PA, 2001; Dokmanovic M, 2005). Moreover, phenylbutyrate has shown only a modest anti-cancer activity in Phase I clinical studies already concluded in leukemias and solid tumors (Dokmanovic M, 2005; Gallinari P, 2007).

SAHA (Zolinza, Vorinostat) is the most clinically advanced HDACi (Hellebrekers DM, 2007; Gallinari P, 2007) approved in 2006 by the Food and Drug Administration (FDA) for the treatment of cutaneous manifestations of advanced, refractory cutaneous T-cell lymphoma (Gallinari P, 2007). SAHA induces differentiation, cell growth arrest and apoptosis in many cell lines at low micromolar concentration and *in vivo* inhibits tumor growth with undetectable toxic effects in a wide range of animal models bearing solid tumors (including breast, prostate, lung and gastric cancers) and hematological malignancies (Gallinari P, 2007). Particularly, SAHA also inhibits the progression of spontaneous tumors that are induced with carcinogens (Johnstone RW, 2002; Marks PA, 2007). To date, many clinical studies with SAHA against different types of tumors have been initiated (Kelly WK, 2003). Intravenous administration of SAHA to patients with hematologic and solid tumors demonstrated that the

drug is well tolerated at the doses required to hyperacetylate histones, and promote symptomatic improvements and tumor regression. The dose limiting toxic effects in hematologic tumors were thrombocytopenia and neutropenia (Johnstone RW, 2002; Minucci S, 2006; Marks PA, 2007). However, inhibition of intracellular HDAC activity needs continuous systemic drug exposure to achieve maximal clinical response. This requirement encouraged development of an oral formulation of SAHA. Phase I study with orally administered Vorinostat in patients with solid and nonsolid neoplasias demonstrated that, in this formulation, SAHA could be administered safely for prolonged periods of time, maintaining the biologic effect of the drug and exhibiting a broad range of antitumor activity (Kelly WK, O'Connor OA, 2005). Toxic effects observed such as fatigue, diarrhea, anorexia and dehydration were reversible on cessation of therapy. Some studies have also demonstrated additive or synergistic effect of SAHA with anticancer agents including chemotherapics (e.g. anthracyclins), DNA demethylating agents (e.g. 5-aza-2'-deoxycytidine), nuclear receptor ligands (e.g. retinoic acid) and apoptotic molecules (e.g. TRAIL ligand) (Marks PA, 2007). Phase I and phase II clinical trials testing SAHA as monotherapy or in combination with different anticancer agents are ongoing for hematologic and solid tumors (Piekarz R, 2004). Phase III studies for SAHA in patients with

malignant pleural mesothelioma and diffuse large B-cell lymphoma are in progress (Hellebrekers DM, 2007). As far as drug-resistance is concerned SAHA is able to reverse the multidrug-resistant phenotype downnregulating P-glycoprotein (Ruefli AA, 2002; Castro-Galache MD, 2003; Peart MJ, 2003).

Depsipeptide (FK-228) is effective in leukaemia and lymphoma animal models, and recently Phase I trials have shown encouraging results for patients with T-cell lymphoma (Hellebrekers DM, 2007). Furthermore, phase II trials are ongoing to study the efficacy and toxicity of FK-228 (Johnstone RW, 2002; Glaser KB, 2007). However, in Phase II studies against solid tumors, FK-228 demonstrated only a marginal activity and many toxic effects such as fatigue, nausea, vomiting, myelosuppression and a significant incidence of cardiac dysrhytmias (Piekarz R, 2004; Minucci S, 2006; Glaser KB, 2007). Nowadays**,** there are several other ongoing Phase II clinical trials testing depsipeptide in patients with renal cell carcinoma, acute myeloid leukaemia, multiple myeloma (Piekarz R, 2004). Although depsipeptide is a substrate for the drug-efflux protein P-glycoprotein, most HDAC inhibitors can overcome multidrug resistance that is mediated by overexpression of P-glycoprotein (Xiao JJ, 2005).

MS-275 inhibits the proliferation of multiple carcinoma cell lines (human leukemia, colorectal, lung, pancreas, ovary and gastric

carcinomas) at micromolar concentration as well as the growth of tumors in mice xenograft models transplanted with the cell lines already tested *in vitro,* in a dose dependent-manner (Saito A, 1999). A phase I study of MS-275 has been performed in patients with advanced solid tumors or lymphoma and this drug is now undergoing Phase II trials (Ryan QC, 2005; Hellebrekers DM, 2007). MS-275 has a half-life in humans longer than that predicted in preclinical models and the dose-limiting toxicities observed in clinical studies are nausea, vomiting, anorexia and fatigue (Minucci S, 2006; Glaser KB, 2007). This drug has potential activity in patients with hematologic malignancies, is currently in clinical trial for melanoma and undergoing Phase II studies in combination with 5-azacitidine in non-small cell lung cancer (Saito A, 1999; Suzuki T,1999; Hauscihld A, 2006; Glaser KB, 2007).

1.11 HDACi mechanism of action

Activation of differentiation programmes, inhibition of the cell cycle and induction of apoptosis are the key antitumor activities of HDACis. These biological effects are due to HDACi-dependent alteration in the acetylation level of histones, transcription factors and other proteins. In fact, the inhibition of HDACs causes the release of these enzymes from transcription factor complexes and the recruitment of HATs which acetylate histones favoring

decondensation of chromatin structure and activation of transcription of tumor-suppressor genes or other genes crucial for the normal functioning of cells (Marks PA, 2001). Although histone deacetylation has a fundamental role in regulating gene expression, HDAC inhibitors seem to directly affect transcription of only a relatively small number of genes (2-10%). Different HDAC inhibitors activate a common set of genes indicating that certain loci are more susceptible to these compounds than others , in particular those that control cell growth and survival (Johnstone RW, 2002). Moreover, HDAC substrates include also non-histone proteins such as transcription factors (e.g. p53, E2F), signal transduction mediator (e.g. STAT3), DNA repair enzyme (e.g. Ku70), chaperone proteins (e.g. Hsp90), structural proteins (α-Tubulin), steroid receptors (ERα). HDACis induce an increase in acetylation level of these proteins affecting their function, stability or protein-protein interaction (Xu WS, 2007; Spange S, 2009). In fact, p53 acetylation increases its stability and binding to DNA leading to transcriptional activation of different p53 target genes including pro-apoptotic genes (e.g. Bax) (Minucci S, 2006). Moreover, acetylation in the DNA-binding domains of Ku70 reduces its ability to repair drug-induced DNA damage, thereby sensitizing cancer cells to agents producing DNA double-strand break as radiations. This effect may account for the

increase of tumor cell sensitivity to chemotherapy and radiotherapy following HDACi treatment (Minucci S, 2006).

One of the main effects of HDACis is inhibition of the cell cycle which is a necessary event in cellular differentiation. Almost all HDACis activate transcription of the cyclin-dependent kinase (CDK) inhibitor p21/WAF1 encoded by the *CDKN1A* gene inducing acetylation of histones at the level of this locus (Nebbioso A, 2005). However, the induction of p15/INK4b (*CDKN2B*), p16/INK4a (*CDKN2A*) and p27/Kip1(*CDKN1B*) CDK inhibitors at transcriptional level has also been reported (Hitomi T, 2003; Marks PA, 2001 Nimmanapalli R, 2003). The increase in the level of p21/WAF1 or other CDK inhibitors causes hypophosphorylation of retinoblastoma tumor-suppressor protein RB and arrest of cell cycle in G1 phase (Johnstone RW, 2002). Analysis of the cell-cycle profiles of tumor cells treated with HDAC inhibitors indicates that the cells often arrest in G1, but sometimes accumulate in G2 phase of cell cycle (Qiu L, 2000). In fact, it has been demonstrated that SAHA at low concentration predominantly induces G1 arrest, while high concentration induces both G1 and G2/M arrests in bladder carcinoma cell lines (Richon VM, 2000). Furthermore, SAHA has been also reported to inhibit the growth of pancreatic cancer cells and non-small cell lung cancer cells (NSCLC) in a dose-dependent manner inducing G2/M cell cycle arrest, apoptosis and differentiation

(Pan LN, 2007). Most tumor cells that do not arrest in G1, after HDACi treatment, replicate their DNA and subsequently arrest in G2 checkpoint (Ruefli AA, 2001; Qiu L, 2000; Glick RD,1999; Johnstone RW, 2002). However, many tumor cell lines accumulating replicated DNA and hyperacetylated histones undergo apoptosis (Johnstone RW, 2002; Bao Q, 2007). In this context, HDACis can induce cell-death of tumor cells activating both mitochondrial (intrinsic) and death-receptor (extrinsic) pathways of apoptosis (Figure 11). The pathway activated depends on the cell type and class of HDAC inhibitor used. The intrinsic apoptosis pathway is usually triggered in response to stress conditions such as cytotoxic drugs, UV irradiation or growth factor withdrawal and requires disruption of the mitochondrial membrane by Bcl-2 family pro-apoptotic members Bax and Bak. Following a death stimulus, the cytosolic monomeric Bax translocates to the mitochondria where it becomes an integral membrane protein and oligomerizes giving rise to voltage-dependent anion channels which lead to mitochondrial outer membrane permeabilization (Johnstone RW, Ruefli AA, 2002; Bao Q, 2007). This event favors the release from the mitochondria of cytochrome c and other pro-apoptotic factors, such as Smac/DIABLO (Second Mitochondria-derived Activator of Caspases). Cytochrome c binds and activates Apaf-1 which in turn activates caspase-9 starting caspase cascade (Johnstone RW,

Ruefli AA, 2002). Smac/DIABLO binds and antagonizes caspase inhibitors IAPs (inhibitors of apoptosis). Caspases are cysteine aspartate proteases expressed as inactive zymogens (pro-caspase) and proteolitically processed to an active state after an apoptotic stimulus. Initiator caspases (e.g. caspase-2, -8, -9 and -10) cleave inactive pro-forms of effector caspases, thereby activating them. Effector caspases (e.g. caspase-3, -6 and -7), in turn, cleave other protein substrates within the cell, to trigger the apoptotic process (Bao Q, 2007).

Bcl-2 family also includes pro-apoptotic Bad and Bim, and antiapoptotic Bcl2 and BclxL proteins. Bad and Bim stimulate activation of intrinsic pathway preventing the antiapoptotic function of Bcl2 and BclxL. HDACis can alter the balance of expression of pro- and anti-apoptotic genes to favor a pro-apoptotic response. Indeed, SAHA causes upregulation of proapoptotic (Bax and Bim) and downregulation of antiapoptotic (IAP, Bcl-2, Bcl-xL) genes. Different HDACis induce also the cleavage of the pro-apoptotic protein Bid resulting in a C-terminal fragment (tBid) which translocates to mitochondria and induces the oligomerization of Bax and Bak opening the mitochondrial voltage-dependent anion channel (Korsmeyer SJ, 2000 ; Johnstone RW, 2003).

The extrinsic pathway of apoptosis is mediated by death receptors including Fas (Apo-1 or CD95), tumor necrosis factor (TNF)

receptor-1 (TNFR-1), TNF-related apoptosis-inducing ligand (TRAIL or Apo2-L) receptors (DR-4 and DR-5). All death receptors are characterized by a cysteine-rich extracellular domain and an intracellular cytoplasmic sequence motif known as the death domain (DD). The binding of Fas-Ligand (Fas-L), TNFα and TRAIL ligands induce trimerization of their receptors and clustering of the receptor DD leading to the recruitment of the adaptor proteins FADD (FAS-Associated Death Domain-containing protein or MORT1) which interacts with the death domain of the receptors through its own death domain. FADD also contains a death effector domain (DED) near its amino terminus, which facilitates binding to the DED of pro-caspase-8 and its activation. This complex (receptor-FADD-pro-caspase-8) of interacting proteins is referred to as the death-inducing signalling complex (DISC) (Johnstone RW, Ruefli AA, 2002; Bao Q, 2007). Pro-caspase-8 is activated by autoproteolytic processing and the resulting active caspase-8 cleaves and activates downstream effector caspases such as caspase-3, -6, and -7. Caspase-8 can also proteolitically activate Bid which can start the mitochondrial pathway amplifying the apoptotic signaling. HDACis can upregulate the expression of both death receptor and their ligands in transformed cells but not in normal cells (Nakata S 2004; Insinga A 2005; Xu WS, 2007). Indeed, it has been reported that Fas and FasL are induced in human neuroblastoma cells by

hydroxamates, in nude mice xenograft of human osteosarcoma cells by FK228 and mouse model of acute promyelocytic leukaemia by valproic acid (Glick RD, 1999; Insinga A, 2005).

TRAIL is induced at mRNA level in acute myeloid leukaemia cells by SAHA and MS-275 and both TRAIL and DR5 are induced in the mouse model of APL by valproic acid (Nebbioso A, 2005; Insinga A, 2005). TNFα is upregulated by FK228 in human promyelocytic leukemia cells (Xu WS, 2007). Therefore, extrinsic apoptotic pathway can account for HDACi-induced cell death in many transformed cells.

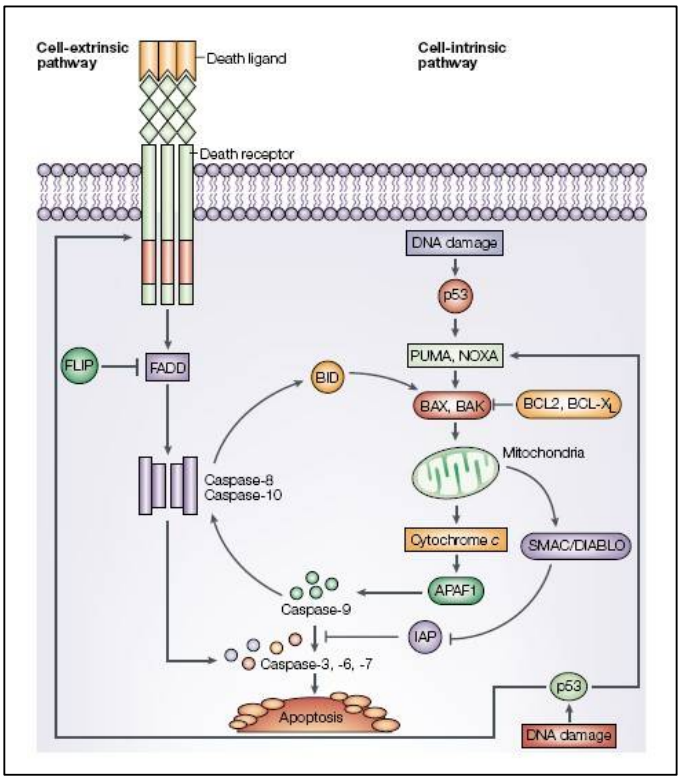


Figure 11. Schematic representation of extrinsic and intrinsic apoptotic pathways

1.12 TNF-related apoptosis-inducing ligand (TRAIL)

TRAIL is one of the main mediators of HDACi-induced apoptosis and as well as a promising candidate for cancer therapeutics due to its ability to induce apoptosis selectively in cancer cells *in vitro* and *in vivo* with little or no effect on normal cells. TRAIL was identified on the basis of its sequence homology to the other members of the TNF superfamily and is expressed as a transmembrane protein of 33 kDa with an extracellular carboxy-terminal and intracellular amino-terminal portion (Ashkenazi A, 2002; MacFarlane M, 2003; Zauli G, 2006; Kawakubo T, 2007). Cell-surface TRAIL protein can be processed by cysteine proteases to release the extracellular carboxy-terminal region (soluble TRAIL) which also leads to a higher induction of apoptosis in tumor cells (Zauli G, 2006; Kawakubo T). TRAIL mRNA is expressed constitutively in a wide range of tissues most predominantly in spleen, lung, and prostate (MacFarlane M, 2003). All cells of the immune system (T-lymphocytes, B-lymphocytes, natural killer cells, dendritic cells, monocytes and granulocytes) can upregulate surface TRAIL expression and/or release soluble TRAIL upon receiving activation signals to mediate anti-tumoral response or maintain the homeostasis of the lymphoid compartment (Zauli G, 2006). Among the TNF superfamily members, TRAIL is the most complex apoptotic ligand as can bind

five different receptors: two death receptors, DR4 and DR5 (also known as TRAIL-R1 and TRAIL-R2), and two decoy receptors, DcR1 and DcR2 (also known as TRAIL-R3 and TRAIL-R4); OPG (osteoprotegerin) is a secreted circulating receptor that also works as a decoy receptor (Ashkenazi A, 2002; MacFarlane M, 2003; Zauli G, 2006). DR4 and DR5 are transmembrane proteins containing a cytoplasmic death domain to elicit an apoptotic response upon binding of TRAIL through DISC complex formation. DcR1 lacks the cytoplasmic portion containing the death domain and is anchored to the cell membrane by a covalently linked C-terminal glycolipid (glycosylphosphatidylinositol). DcR2 is a transmembrane protein with a truncated death domain in its cytoplasmic tail (MacFarlane M, 2003; Zauli G, 2006). Both DcR1 and DcR2 are unable to efficiently transduce intracellular apoptotic signals and may compete with DR4 and DR5 for ligand TRAIL thus working as decoy receptors. TRAIL receptors are expressed in most human tissues including spleen, thymus, peripheral blood lymphocytes and gastrointestinal tract (Pan G, 1997; Schneider P, 1997; Ashkenazi A, 2002; Mahalingam D, 2008). OPG acts in a paracrine and autocrine manner by binding TRAIL, preventing its access to DR4 and DR5 promoting cell survival (Zauli G, 2006). In addition to triggering a pro-apoptotic signal through activation of caspases, the binding of TRAIL to death receptors DR4 and DR5, can activate an alternative signal

transduction pathway which promotes proliferation and survival through NF-κB (nuclear factor κB) transcription factor (Johnstone RW, 2008). Indeed, following ligand binding, TRAIL death receptors can recruit FADD directly or TRADD (TNFR-associated death domain) adaptor which in turn recruits FADD to start the apoptotic cascade. However, TRADD can also recruit TRAF2 (TNF receptor-associated factor 2) and RIP (receptor interacting protein) which succesively recruit IKK (κB kinase complex) favoring phosphorylation of IκBα (NF-κB inhibitor) and signaling it for ubiquitination and degradation (Chaudhary PM, 1997). These events allow NF-κB to enter the nucleus and activate transcription of pro-survival genes such as cytokines or growth factors (MacFarlane M, 2003). TRAIL seems to activate NF-κB only when the apoptotic signalling is blocked by use of caspase inhibitor or via endogenous resistance mechanisms also due to defective death receptor signaling found in certain tumor cells (Ehrhardt H, 2003; Baader E, 2005; Johnstone RW, 2008).

Recombinant soluble TRAIL induces apoptosis in a broad spectrum of human cancer cell lines, including colon, lung, breast, prostate, pancreas, kidney, central nervous system, and thyroid cancer, as well as multiple myeloma, melanoma and osteosarcoma indicating that this ligand might be useful for the treatment of many cancers

(Ashkenazi A, 2002; Zauli G 2006; Wu GS, 2009; Macher-Goeppinger S, 2009). Furthermore, unike TNFα and Fas-L which are toxic upon systemic administration in athymic mice, injection of recombinant TRAIL exerts marked antitumor activity without toxicity in both mice and non-human primates (Ashkenazi A, 2002; Johnstone RW, 2008). HDACIs strongly sensitized exogenous soluble recombinant human TRAIL-induced apoptosis synergistically in different tumor cell lines that were resistant to TRAIL alone (Bolden JE, 2006; Earel JK, 2006; Newsom-Davis T, 2009). Therefore, the recombinant form of TRAIL has recently entered a Phase I clinical trial as monotherapy or in combination with chemo, HDAC inhibitors or other anticancer drugs (Johnstone RW, 2008).

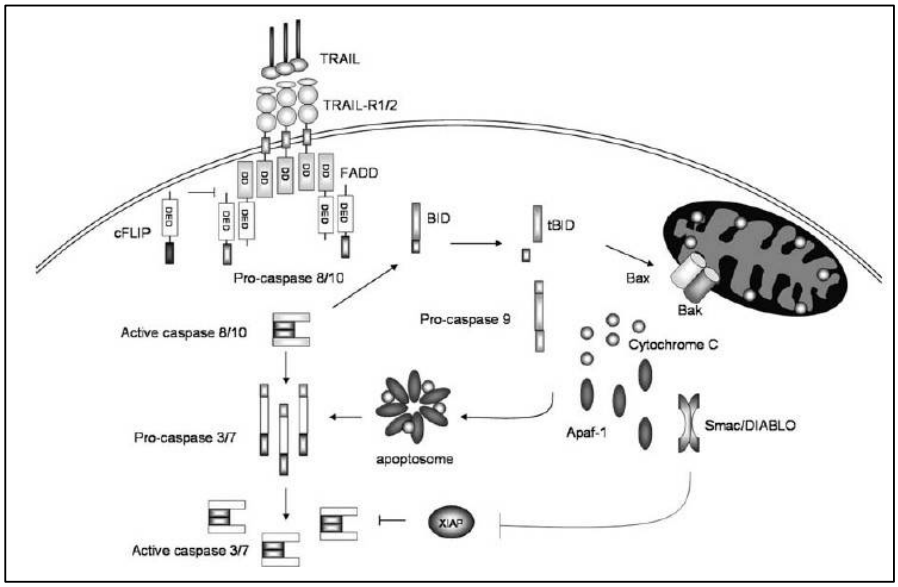


Figure 12. Apoptotic pathways activated by TRAIL ligand.

Aim of the project

The aim of our work has been to evaluate the possibility of an innovative therapy for anaplastic thyroid carcinoma based on HDACis, investigating the effects and mechanisms of action of some HDACis on a model of rat thyroid cells transformed by the *v-ras*-Ki oncogene, which is one of the most common genetic lesions found in human tumors. We have chosen SAHA (suberoylanilide hydroxamic acid), and MS-275 (Mitsui), two potent HDACis in clinical trial for the treatment of different types of tumors

2 MATERIALS AND METHODS

2.1 Immunohistochemistry

Immunohistochemical analysis for HDAC1, HDAC2, HDAC3 proteins was performed by Dr. Gennaro Chiappetta (National Cancer Institute G. Pascale, Naples). Paraffin sections (5 to 6 μm) were deparaffinized, placed in a solution of absolute methanol and 0.3% hydrogen peroxide for 30 minutes and then washed in PBS before immunoperoxidase staining. For HDAC1, HDAC2 and HDAC3 slides were incubated overnight at 4°C in a humidified chamber with HDAC 1 rabbit polyclonal antibody (Cell Signaling Technology) 1:50; HDAC 2 polyclonal antibody (Santa Cruz Biotechnology) 1:50; HDAC 3 rabbit polyclonal antibody (Cell Signaling Technology) 1:50 dilution. The slides were subsequently incubated with biotinylated goat antirabbit/antimouse IgG for 20 minutes (DakoLSAB2 System) and then with streptavidin horseradish peroxidase for 20 minutes. For immunostaining, the slides were incubated in 3,3'-diaminobenzidine (Dako) solution containing 0.06 mmol/L 3,3'-diaminobenzidine and 2 mmol/L hydrogen peroxide in 0.05% PBS (pH 7.6) for 5 minutes. After chromogen development, the slides were washed, dehydrated with alcohol and xylene, and mounted with coverslips with Permount mounting medium (Proscitech, Kirwan, Australia). *The informed*

consent for the scientific use of biological material was obtained from all patients.

2.2 Cell lines

FRTL-5 Cl2 and PC Cl3 are thyroid epithelial cell lines derived from 3-week- and 18-month-old Fisher rats, respectively. These cell lines are not tumorigenic and retain the typical markers of thyroid differentiation (thyroglobulin synthesis and secretion, iodide uptake and dependance on TSH) (Fusco A ,1985; Fusco A, 1987). FRTL-5 *v-ras*-Ki, PC Cl3 *v-ras*-Ki, are rat thyroid cell lines obtained from FRTL-5 Cl2 and PC Cl3 infected with Kirsten murine sarcoma virus (KiMSV) carrying *ki-ras* oncogene. FRTL-5 *v-ras*-Ki cells have lost the typical markers of thyroid differentiation and are completely transformed because they grow with high efficiency in agar and induce tumors after brief latency period (7-10 days) after injection in athymic mice. PC Cl3 *v-ras*-Ki lost the typical markers of thyroid differentiation (thyroglobulin synthesis and secretion, iodide uptake and dependance on TSH) but do not became fully malignantly transformed. PC Cl3 *v-mos* are rat thyroid cell lines derived from PC Cl3 infected with myeloproliferative sarcoma virus (MPSV) carrying *mos* oncogene and are not differentiated and they are fully transformed (Fusco A ,1985; Fusco A, 1987). All rat thyroid cell lines

were cultured in Coon's modified Ham's F12 medium supplemented with 5% calf serum, 1% penicillin/streptomycin (Gibco Laboratories, USA). Normal FRTL-5 Cl2 and PC Cl3 cells required 6 growth factors ($1x10^{-10}$ M TSH, 10 µg/ml insulin, 10 nM hydrocortisone, 5 µg/ml human transferring, 10ng/ml somatostatin, 10ng/ml glycyl-L-histidyl-L-lysine acetate). FRO (Fagin JA, 1993) and FB1 (Fiore L, 1997) are human thyroid anaplastic carcinoma cell lines. They were grown in DMEM (Gibco Laboratories) containing 10% FCS (Gibco Laboratories), 1% L-glutamine (Gibco Laboratories) and 1% penicillin/streptomycin (Gibco Laboratories). All cell lines were grown at 37°C in a 5% CO2 atmosphere.

2.3 Chemicals

MS-275 (kind gift from Bayer-Schering) and MG132 (Calbiochem) were dissolved in ethanol while SAHA (Alexis) and MC1568 in dimethylsulfoxide (DMSO).

2.4 RNA and protein extraction

FRTL-5 Cl2 and FRTL-5 *v-ras*-Ki cells were plated in 10 cm dishes and treated with 5 µM SAHA and 5 µM MS-275 for 24, 48 and 72 hours. RNA and protein were extracted as described below.

2.4.1 RNA extraction

RNA was extracted with TRIzol (Invitrogen). Cells were homogenized in 1 mL TRIzol solution and 200 µL of chloroform were added. The resulting mixture was vortexed for 15 seconds, incubated at room temperature for 15 minutes and centrifugated at 11000 rpm for 15 minutes at 4°C. RNA contained into the aqueous phase was precipitated with 500 µL of isopropanol for 10 minutes at room temperature. The precipitate was centrifugated at 11000 rpm for 8 minutes at 4°C and the pellet obtained was washed with 1mL of 75% ethanol and centrifugated at 8000 rpm for 5 minutes at 4°C. After removal of supernatant the pellet was air-dried at room temperature and dissolved in RNase-free water (DEPC-treated water, Ambion). RNA concentration was measured using a Nanodrop spectrophotometer (Thermo Scientific, USA).

2.4.2 Protein extraction

FRTL-5 Cl2 and FRTL-5 *v-ras*-Ki cells were treated with 5 µM SAHA and 5 µM MS-275, harvested and whole cell protein extracts were prepared as further described. Cells were washed once in cold Phosphate Buffered Saline (PBS) and lysed in a lysis buffer containing 50mM Tris-HCl pH 7.4, 150mM NaCl, 1% NP40, 0.25% sodium deoxycholate, 1mM EDTA, 1mM phenylmethyl-sulphonyl-

fluoride and protease inhibitor cocktail. The lysates were centrifugated at 14000 r.p.m. x 30 min at 4°C. Protein concentrations were estimated by Bradford assay (Bio-Rad, Italy).

2.5 Reverse transcription (RT) and Polymerase Chain Reaction (PCR)

RNA was reverse transcribed in cDNA using Qiagen QuantiTect Reverse Transcription kit (Qiagen, Germany). Polymerase Chain Reaction was performed to amplify TRAIL and β-actin or GAPDH normalizators cDNAs using HotMaster Taq DNA Polymerase (Eppendorf, Germany). The thermal protocol used for TRAIL amplification was as follows: initial denaturation at 94°C for 2 min followed by 35 cycles of amplification using a thermal cycle program consisting of 94°C for 20 s of denaturation, 57°C for 45 s annealing, 65°C for 45 s of extension and 65°C for 2 min of final extension.

The primers used were as follows:

Name	**Sequence**	**Amplicon length** (bp)
Rat TRAIL forward	5'-GCTTCAGTCAGCACTTCACG-3'	179
Rat TRAIL reverse	5'-GTCCCAAAAATCCCCATCTT-3'	
Rat G6PDH forward	5'-ACAGAGTGAGCCCTTCTTCAA-3'	106
Rat G6PDH reverse	5'-GGAGGCTGCATCATCGTACT-3'	
Rat β-actin forward	5'-TCGTGCGTGACATTAAGGAG-3'	110
Rat β-actin reverse	5'-GTCAGGCAGCTCGTAGCTCT-3'	

2.6 Western blot analysis

Protein were denaturated boiling samples in Laemmli buffer (0.25 M Tris-HCl pH 6.8, 8% SDS, 40% glycerol, 5% 2-mercaptoethanol, bromophenol blue 0.05%) for 5 min before electrophoresis. Proteins were subjected to SDS–PAGE (12-15% polyacrylamide) in Tris-glycine-SDS (25 mM Tris, 192 mM glycine, 0.1% SDS) (Bio-Rad, Italy). After electrophoresis, proteins were transferred to nitrocellulose membranes (Schleicher e Schuell, Germany) in a buffer containing Tris-glycine (25 mM Tris, 192 mM glycine) (Bio-Rad, Italy) and 20% methanol. The complete transfer was assessed using Ponceau red (Sigma Aldrich, USA) staining. After blocking with 5% non-fat dry milk in TBS 1x/Tween 0.1% (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% Tween-20), the membrane was incubated with the primary antibody overnight at 4°C. After washing with TBS 1x/Tween 0.1%, membranes were incubated with the horseradish peroxidase-conjugated secondary antibody (1:3000) for 60 min at room temperature and the reaction was detected with chemioluminescence detection system (Amersham Biosciences, UK). The antibodies used were as follows: Acetylated-H3 (Ac-H3) (Upstate Biotechnology, USA); p27/Kip1 (BD Transduction laboratories, USA); Bcl-2 (Stressgen, USA); p21/Waf-1, Bax, HDAC2 and GAPDH (Santa Cruz Biotechnology, USA); HDAC1,

HDAC3 and ERK1/2 (Cell signaling, USA); BclxL (R&D, USA), TRAIL (Chemicon, USA); α-Tubulin (Sigma Aldrich, USA).

2.7 RNase protection assay

The Ribonuclease (RNase) Protection Assay was performed by Professor Lucia Altucci research group (Seconda Università degli Studi di Napoli, Naples) according to standard procedures (Pharmingen, San Diego, California) (Altucci L, 2001; Scognamiglio, A, 2008). RNA of rat thyroid cell lines used for this analysis was extracted as described above. For hybridization 6 µg of total RNA and 6-8 $x10^5$ cpm/µl of labeling probe were used. These fragments were recovered by ethanol precipitation and analyzed by electrophoresis on a sequencing gel (5% of urea-polyacrylamide-bis-acrylamide). After the drying process, the gel was placed in contact with an autoradiographic film (Hyperfilm-MP, Amersham). The exposure time was overnight. The presence of the target mRNA in the samples was revealed by the appearance of an appropriately sized fragment of the probe.

2.8 Detection of apoptosis and cell cycle analysis

FRTL-5 Cl2, FRTL-5 *v-ras*-Ki, PC Cl3, PC Cl3 *v-ras*-Ki and PC Cl3 *v-mos* cell lines were treated with 5 μM SAHA and 5 μM MS-275 for 24, 48 and 72 hours. FRTL-5 Cl2 and FRTL-5 *v-ras*-Ki, were also treated with 5 μM MC1568 for 24, 48 and 72 hours. Apoptosis was quantified by propidium iodide-Annexin V double staining (Altucci L, 2001). For recycling experiments: after treatment of FRTL-5 Cl2 and FRTL-5 *v-ras*-Ki cells with SAHA and MS-275, the medium was replaced with fresh medium and cells remained in culture for additional 24 and 48 hours. Then, cells were collected and cell cycle was analyzed by FACS (fluorescence-activated cell sorting).

FACS analysis: 2.5 x 10^5 cells were collected and resuspended in 500 μL of hypotonic buffer (0.1% Triton X-100, 0.1% sodium citrate, 50 μg/mL propidium iodide, RNase A). Cells were incubated in the dark for 30 min. Samples were acquired on a FACS-Calibur flow cytometer using the Cell Quest software (Becton Dickinson) and analyzed by standard procedures using the same software and the ModFit LT version 3 Software (Verity) (Nebbioso A, 2005; Scognamiglio A, 2008). Acquisition and analysis of FACS data was performed by Professor Lucia Altucci (Seconda Università degli Studi di Napoli, Naples).

2.9 Caspase assay

FRTL-5 Cl2 and FRTL-5 *v-ras*-Ki were treated with 5 μM SAHA and 5 μM MS-275 for 24 hours and then collected. Cell pellet was resuspended in 300 μL of a solution containing a caspase substrate (B-Bridge) diluted in PBS 1X. Then, the samples were incubated at 37°C for 1h. After the reaction was concluded cell samples were washed, resuspended in 500 μL of Wash buffer (B-Bridge) and caspase-3, -8 and -9 activities were measured by FACS (Bontempo P, 2007). Caspase substrates (B-Bridge, Kit) used consist of: a different peptide sequence which is a specific target for each casapase bound to a fluorochrome and to fluoromethyl ketone, a potent inhibitor of caspase activity. These substrates enter each cell and irreversibly bind to a reactive cysteine residue that resides on the large subunit of the active caspase, thereby inhibiting further enzymatic activity. The fluorescent signal is a direct measure of the number of active caspase enzymes present in the cell.

2.10 RNA interference

siRNAs for rat TRAIL and for negative control (All stars negative siRNA) were purchased from Qiagen. The sense and antisense strands of TRAIL siRNA were: 5'-PCAACGAGGUGAAACAGCUAdT dT-3'(sense),5'-PUAGCUGUUUCACCUCGUUGdTdT3'(anti-sense);

P represents 5' phosphate. The sense strand of both TRAIL siRNA and the negative control siRNA were labeled with Alexa Fluor 488 at 3'end. FRTL-5 Cl2 and FRTL-5 *v-ras*-Ki cells were transfected with siRNAs (15 nM) using Hiperfect transfection reagent (Qiagen, Germany) and treated with 5 μM SAHA for 24 hours. After treatment, the cells were collected and analyzed by FACS to measure both efficiency of transfection and caspase-3 activity within the transfected cells. The efficiency of TRAIL knock-down was evaluated by RT-PCR and analysis of cell-surface TRAIL protein levels by using an anti-TRAIL-PE (E-bioscience) antibody FACS and Cell Quest analyses.

2.11 Drug combination studies

FRTL-5 Cl2 and FRTL-5 *v-ras*-Ki were treated with 1 μM MG132 for 3 hours and then with 1 μM or 2.5 μM SAHA for 24 hours. SAHA and MG132 were also combined at two different concentrations: 2.5 μM SAHA plus 1 μM MG132 or 1 μM SAHA plus 1 μM MG132. FRO and FB1 cells were treated with 0.25 μM or 2 μM MG132, respectively, for 3 hours and then with 5 μM SAHA for 24 hours. After treatment, cells were collected and analyzed by FACS.

2.12 Protein stability

To study the half-life of TRAIL protein, FRTL-5 Cl2 and FRTL-5 *v-ras*-Ki were treated with 5 μM SAHA for 24 hours and 5 μg/ml cycloheximide (translation inhibitor) for 2, 4 and 8 hours. After treatment, cells were harvested and protein extracts were analyzed by Western blot.

2.13 Degradation assay

FRTL-5 *v-ras*-Ki were co-transfected with GFP (pEGFP-N1) (Clontech) and histidine-tagged ubiquitin (p-Ubi-His) plasmids or with TRAIL-GFP fusion protein (pEGFP-TRAIL) (Addgene) and histidine-tagged ubiquitin (p-Ubi-His) plasmids using Fugene HD reagent (Roche). After transfection, cells were treated with 5 μM SAHA for 16 hours, collected and analyzed by FACS.

3 RESULTS

3.1 HDAC expression in human thyroid tumor biopsies and in rat thyroid cell lines

First, we evaluated the HDAC 1, 2 and 3 protein levels in normal thyroid, in papillary (PTC), follicular (FTC) and anaplastic (ATC) thyroid carcinomas by immunohistochemistry. HDAC 1 and 2 proteins were found highly overexpressed in ATC samples when compared to normal thyroid tissue. The immunohistochemical results are summarized in Figure 13, A-H. An intense nuclear staining for HDAC1 and HDAC2 was observed in the ATCs (Figure 13, B-H), with respect to normal thyroid (Figure 13, A and E); conversely, HDAC3 staining did not show significant differences in ATCs compared to papillary and follicular carcinomas as well as to normal thyroid tissue (not shown). Thus the increase in HDAC 1 and 2 expression positively correlates with malignancy (Table 2).

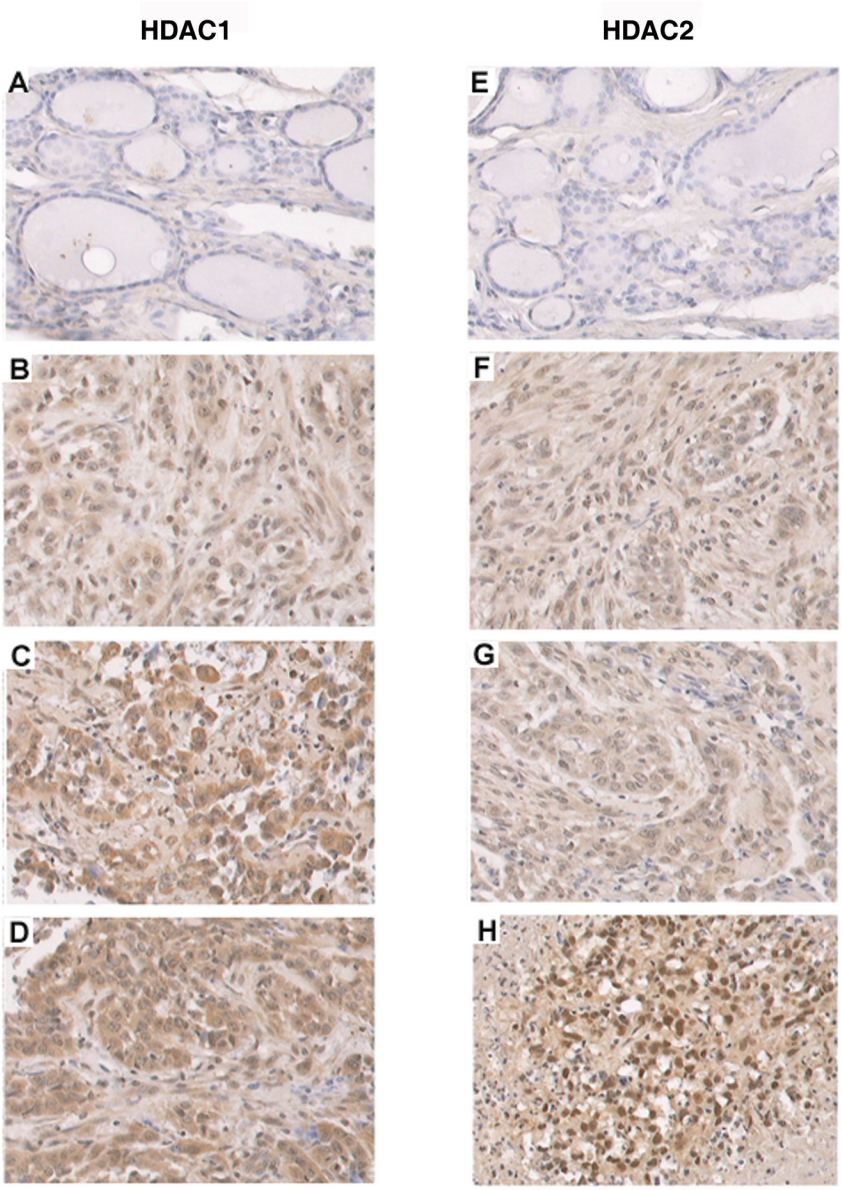


Figure 13. Immunohistochemical detection of HDAC 1 and 2 in human thyroid tumors. (A and E) Immunostaining of a normal thyroid. (B-H) Immunostaining of anaplastic thyroid carcinomas.

Histotype	Cases	Positive cases*	
		HDAC1	HDAC2
Papillary carcinomas	17	47%	41%
Anaplastic carcinomas	11	55%	63%

*Positive cases with more than 50% of neoplastic cells showing HDAC staining

Table 2. Papillary and Anaplastic thyroid carcinoma positive cases to HDAC1 and 2 staining by immunohistochemistry

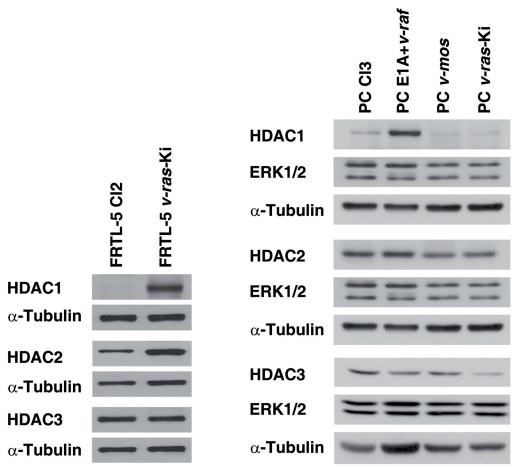
Subsequently, we analyzed class I HDAC expression in normal and transformed rat thyroid cell lines. The characteristics of the cell lines used are summarized in Figure 14A. HDAC1 protein levels were higher in transformed cells (FRTL-5 *v-ras*-Ki and PC E1A+*v-raf*) than in normal cells (FRTL-5 Cl2 and PC Cl 3). HDAC2 protein was more abundant in FRTL-5 *v-ras*-Ki than in FRTL-5 Cl2. No significant differences in HDAC3 expression levels between normal and transformed cells were observed (Figure 14B). The expression of the specific HDAC 1, 2, and 3 mRNA, analyzed by RNase protection assay, essentially supported the data on protein levels indicating that the expression of class I HDACs is regulated at transcriptional level and that HDAC 1 and 2 are overexpressed in malignant thyroid cells (Figure 14C).

A

Group	Cell type	Oncogene	Differentiation status
Nontumorigenic cell lines*	PC Cl3		Differentiated
	PC *v-ras*-Ki	*v-ras*-Ki	Undifferentiated
	PC *v-ras*-Ha	*v-ras*-Ha	Undifferentiated
	PC *v-raf*	*v-raf*	Undifferentiated
	PC HMGA2 a-s+*v-mos*	HMGA2 a-s+*v-mos*	Undifferentiated
	PC PTC-1	RET/PTC1	Undifferentiated
	FRTL-5 Cl2		Differentiated
Lowly tumorigenic cell lines†	PC Py MLV	Polyomavirus middle T	Differentiated
Highly tumorigenic cell lines‡	PC E1A+*v-raf*	E1A+*v-raf*	Undifferentiated
	PC *v-mos*	*v-mos*	Undifferentiated
	FRTL-5 *v-ras*-Ki	*v-ras*-Ki	Undifferentiated

* No tumors appeared after injection of 2 x 10^6 cells into six athymic mice
† Tumors appeared at least 3 weeks after injection of 2 x 10^6 cells into athymic mice
‡ Tumors appeared not later than 10 days after injection of 2 x 10^6 cells into athymic mice

B



C

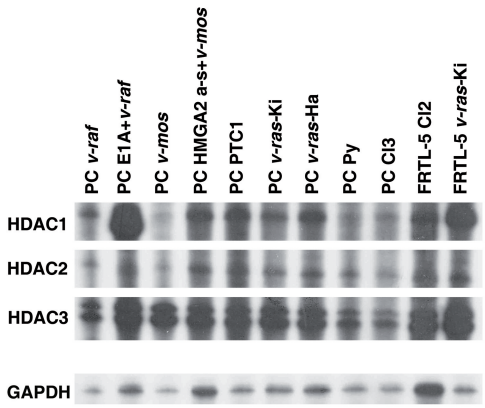


Figure 14. Analysis of HDAC 1, 2 and 3 expression in rat thyroid cell lines. (A) Characteristics of normal and transformed rat thyroid cell lines. (B) Analysis of HDAC protein levels by Western blot. α-Tubilin and ERK1/2 have been used as controls for equal protein loading. (C) HDAC mRNA expression analysis by RNase protection assay in different rat thyroid cell lines. GAPDH mRNA has been used as control for equal mRNA loading.

3.2 SAHA and MS-275 induce growth arrest and apoptosis in *v-ras*-Ki transformed rat thyroid cells.

To test whether the overexpression of HDAC 1 and 2 corresponds to the increased effect of HDACis in tumor thyroid cells, we treated normal (FRTL-5 Cl2 and PC Cl 3) and transformed (FRTL-5 *v-ras*-Ki, PC Cl 3 *v-ras*-Ki, PC Cl 3 *v-mos*) rat thyroid cell lines with 5 μM SAHA or 5 μM MS-275 for 24, 48 and 72 hours. Then, we analysed cell cycle and apoptosis by FACS.

3.2.1 Analysis of HDACi-induced apoptotic effect

FACS analysis in Figure 15 shows that both SAHA and MS-275 induced apoptosis preferentially in the transformed cell lines. In fact, the apoptotic effect was more evident in the highly tumorigenic cell lines such as FRTL-5 *v-ras*-Ki and PC Cl 3 *v-mos* (Figure 15B) than in PC Cl 3 *v-ras*-Ki which did not show a malignant phenotype (Figure 15C), thus suggesting that HDACis might be more efficient in highly malignant thyroid tumors. Normal cell lines FRTL-5 Cl2 and PC Cl3 (Figure 15A), did not show any apoptotic effect after treatment. In full agreement with these findings, caspase-3 (effector caspase) and caspase-8, -9 (initiator caspases) were activated in FRTL-5 *v-ras*-Ki but not in FRTL-5 Cl2 (Figure 15D), thus indicating that caspase-dependent apoptosis mediates cell death in transformed cells but not in normal thyroid cells.

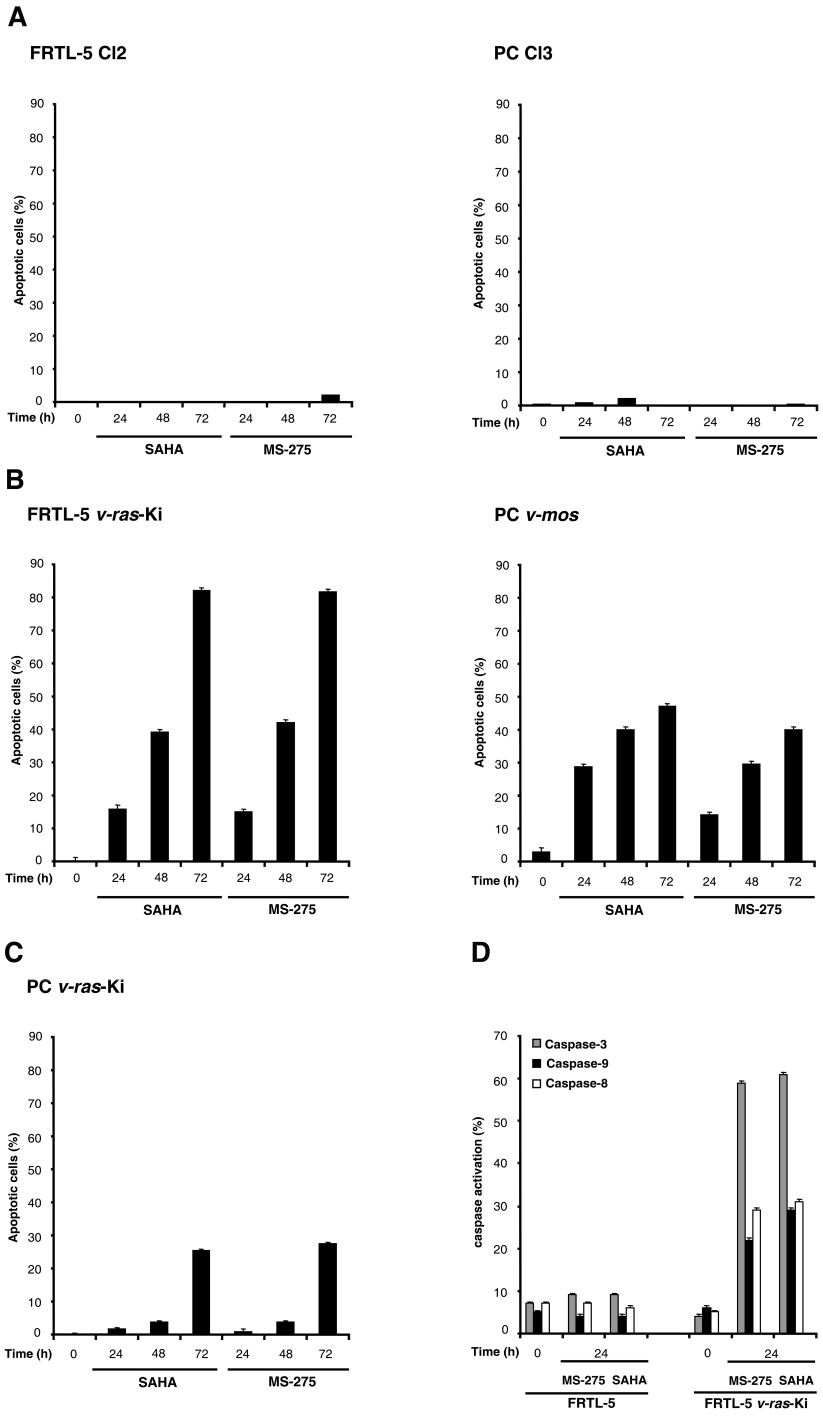


Figure 15. SAHA and MS-275 induce apoptosis in transformed thyroid cells. FACS analysis of normal (A) and transformed (B and C) rat thyroid cells treated with SAHA and MS-275 for 24, 48 and 72 hours. (D) FACS analysis of FRTL-5 Cl2 and FRTL-5 *v-ras*-Ki treated for 24 hours with SAHA and MS-275 to measure caspase -3,-8 and -9 activation.

3.2.2 Analysis of HDACi-induced cell cycle arrest

In a similar experimental setting, the cell cycle analysis did not show significant differences between tumor and normal cell lines (Figure 16, A and B). Moreover, both SAHA and MS-275 vehicles (DMSO and ethanol respectively) did not affect cell cycle or apoptosis of the analyzed cell lines at the concentration used. Furthermore, the evaluation of histone H3 acetylation by Western blot analysis (Figure 16C) did not display significant differences of acetylated histone regulation among FRTL-5 Cl2 and their tumoral *ras* version. Indeed, we could detect an increase of H3 acetylation and a cell cycle arrest in both normal and cancer cells.

However, to evaluate the extent of SAHA and MS-275 effects in these thyroid cell lines, we treated FRTL-5 Cl2 and FRTL-5 *v-ras*-Ki cell lines as previously described, removing the drugs and allowing the cells to grow for additional 24 and 48 hours. As shown in Figure 16D, FRTL-5 Cl2 re-entered the S phase after drug removal, while FRTL-5 *v-ras*-Ki treated for 72 hours, but not those treated for 24 and 48 hours (not shown), remained blocked in G1 phase going towards death. These results, corroborate the low toxicity of SAHA and MS-275 for normal thyroid cells, thus stressing the selectively induced cell death caused by HDAC inhibitors in transformed cells.

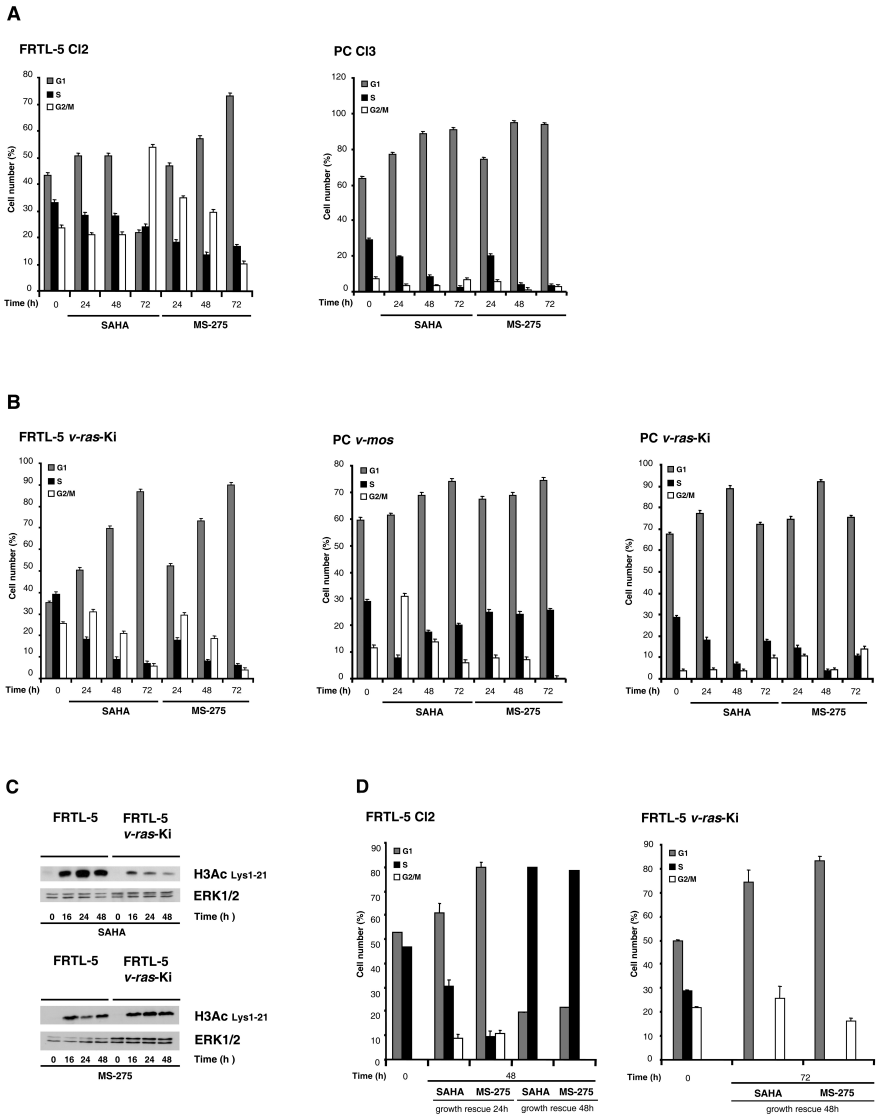


Figure 16. SAHA and MS-275 induce growth arrest in rat thyroid cells. (A and B) FACS analysis of normal and transformed cells treated for 24,48 and 72 hours with SAHA and MS-275.(C) Western blot analysis of acetylated histone H3 in both FRTL-5 and FRTL-5 *v-ras*-Ki treated with SAHA and MS-275.ERK1/2 has been used as control for equal protein loading. (D) Cell cycle analysis performed by FACS of FRTL-5 and FRTL-5 *v-ras*-Ki treated with SAHA and MS-275 for 48 and 72 hours and grown after removal of both drugs.

3.2.3 Inhibition of Class I HDACs is crucial for HDACi-Induced apoptosis and cell cycle arrest

Finally, the compound MC1568, which is known to inhibit only class II HDACs (Mai A, 2005; Duong V, 2008) (SAHA and MS-275 inhibit also or only class I HDACs), induced only a slight G1 arrest, but did not cause apoptosis in both FRTL-5 Cl2 and FRTL-5 *v-ras*-Ki (Figure 17). This evidence suggests that the apoptotic effects induced by SAHA and MS-275 in the FRTL-5 *v-ras*-Ki cells are due to the inhibition of class I HDACs and, in particular, to the block of HDAC 1 and 2, the only HDACs mainly inhibited by MS-275 (Nebbioso A, 2005) and found overexpressed in thyroid carcinomas (Figure 13).

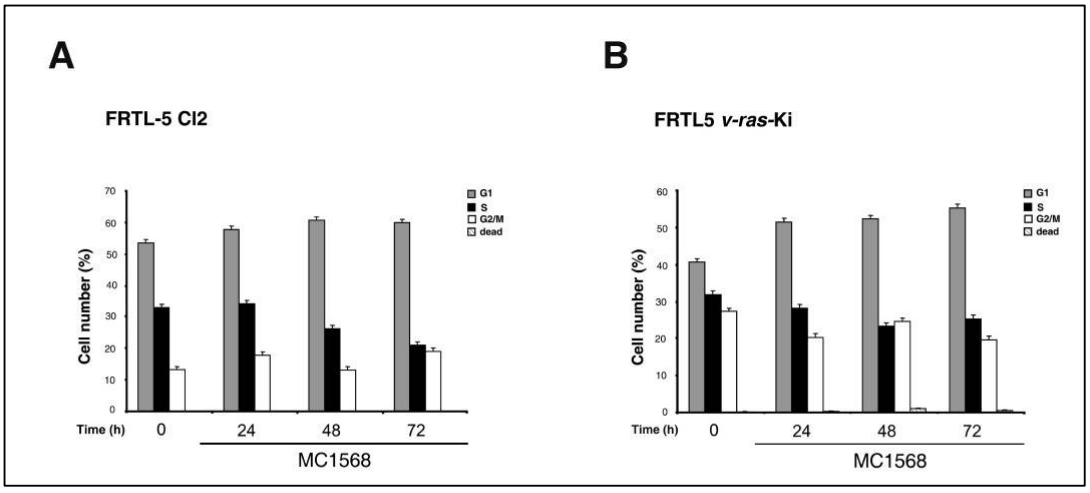


Figure 17. MC1568 does not induce apoptosis in rat thyroid cells.
(A and B) FACS analysis of FRTL-5 and FRTL-5 *v-ras*-Ki treated with a class II HDAC inhibitor (MC1568) for 24, 48 and 72 hours.

3.3 SAHA and MS-275 alter regulatory protein levels in FRTL-5 *v-ras*-Ki thyroid cells

In order to understand the molecular events occurring upon HDACi treatment and underlying to the selective tumor cell death, we evaluated the levels of known proteins involved in apoptosis such as Bax, Bcl2, BclxL, in both FRTL-5 Cl2 and FRTL-5 *v-ras*-Ki before and after treatment. The treatment with both HDACis did not affect the protein levels of Bax and Bcl2, whereas BclxL was up-regulated in normal and down-regulated in cancer cells (Figure 18). High levels of BclxL could be important in blocking the apoptotic process in normal cells. When cell cycle related proteins were investigated, p21/WAF1 was induced only by MS-275 in both normal and transformed cells, whereas p27/Kip1 was induced by both drugs only in normal cells. Therefore, it is reasonable to speculate that p27 induction might be responsible for the cell cycle arrest caused by these HDACis in normal thyroid cells.

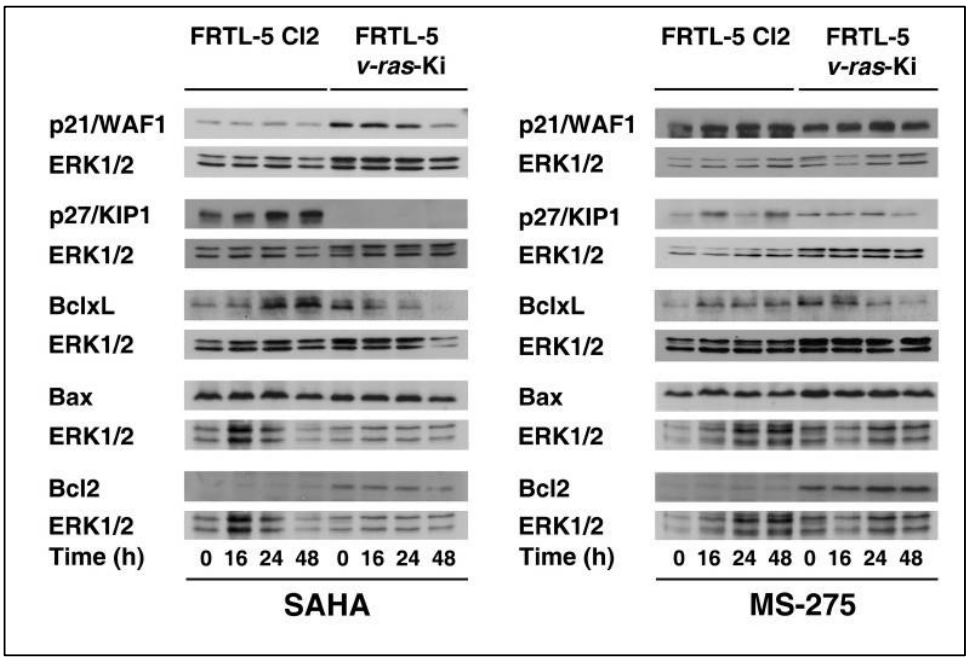


Figure 18. Western blot analysis of proteins regulating cell cycle and apoptosis in FRTL-5 and FRTL-5 *v-ras*-Ki treated with SAHA and MS-275 for 16, 24 and 48 hours. Blot against ERK1/2 has been performed as control for equal protein loading

It has previously been demonstrated that TRAIL (TNFSF10, Apo2L, TNFSF10-Tumor necrosis factor ligand superfamily member 10) is induced by HDACis (Nebbioso A, 2005) and that its action displays some tumor selective feature (Hall MA, 2007; Johnstone RW, 2008) although the mechanisms for this remain presently obscure. Therefore, we analyzed TRAIL expression by Western blot in normal and transformed cells. As shown in Figure 19 both SAHA and MS-275 induced TRAIL protein only in FRTL-5 *v-ras*-Ki cells. Although TRAIL regulation at transcriptional level has previously been described (Nebbioso A, 2005), in our system, RT-PCR analysis did not show significant differences between treated and untreated,

normal and transformed cells in TRAIL mRNA expression level (Figure 19B), thus suggesting that a different regulation is occurring.

3.4 TRAIL is the main mediator of cell death induced by SAHA

Intrigued by the TRAIL induction stimulated by HDACis in tumor thyroid cells, we transiently knocked down TRAIL by an interference methodology in FRTL-5 Cl2 and FRTL-5 *v-ras*-Ki cell lines. The TRAIL mRNA expression was drastically reduced in the siTRAIL transfected cells as the efficiency of TRAIL knock-down, evaluated by RT-PCR and TRAIL protein cell-surface level analysis (Figure 19C), clearly demonstrates. Interestingly, TRAIL silencing caused a significant reduction (61%) in caspase-3 activation in FRTL-5 *v-ras*-Ki compared to the same SAHA-treated cells transfected with a scramble siRNA (Figure 19D). The analysis of caspase-3 performed only in the transfected cells ensured that the decrease in apoptosis occurred for TRAIL silencing. This result indicates that TRAIL is a main mediator of the apoptosis induced by HDACis in transformed thyroid cells.

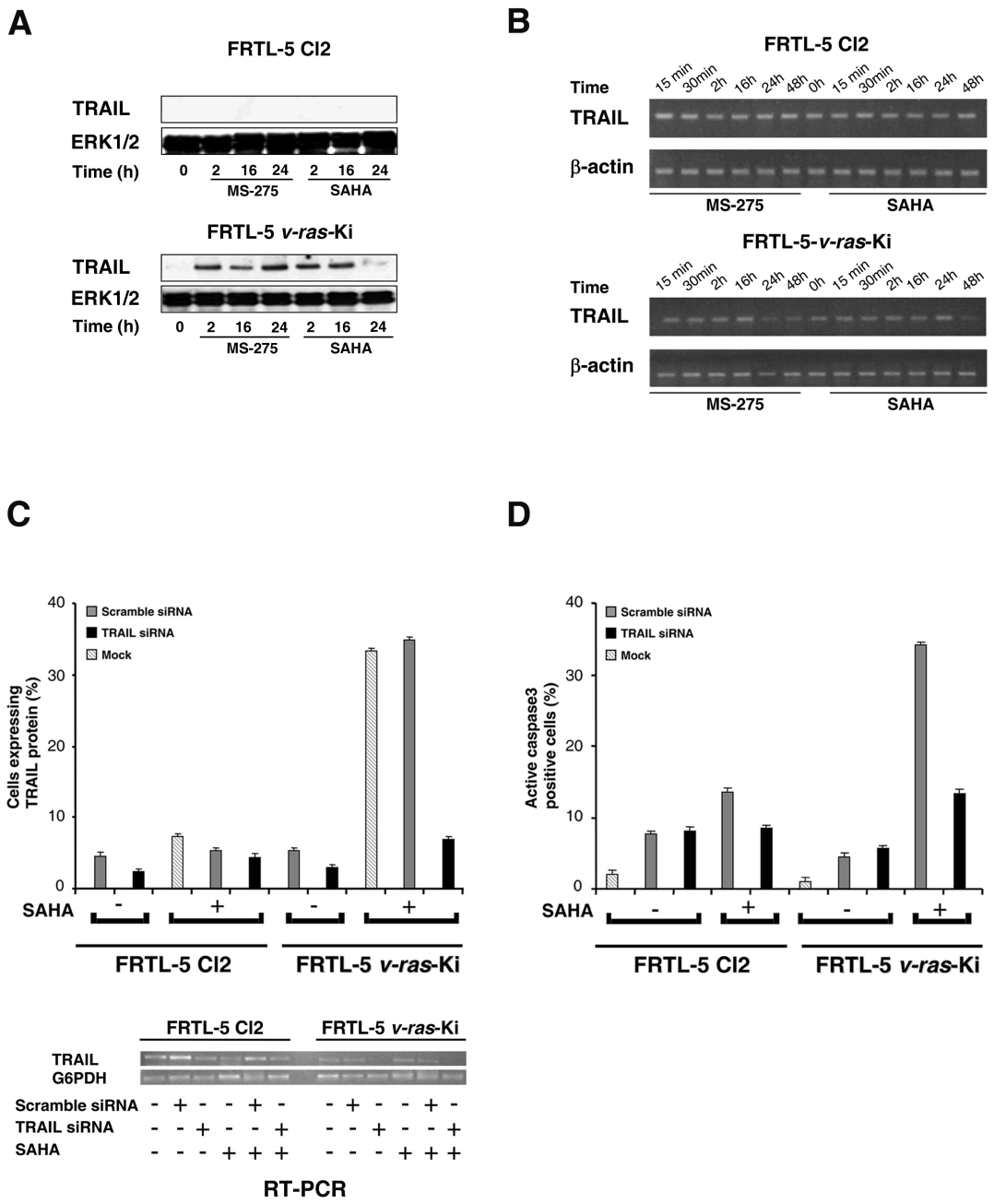


Figure 19. Expression analysis of death ligand TRAIL in normal and transformed rat thyroid cell lines. (A and B) Western blot and RT-PCR analysis of TRAIL expression at protein (A) and mRNA level (B), in FRTL-5 and FRTL-5 *v-ras*-Ki cells treated with SAHA and MS-275. ERK1/2 and β-actin have been used as control to normalize the amount of used proteins and RNA, respectively. (C) RT-PCR (bottom) and FACS (top) analyses of TRAIL mRNA and cell-surface protein levels in FRTL-5 and FRTL-5 *v-ras*-Ki cells transfected with scramble or TRAIL siRNAs and treated with SAHA. G6PDH has been used as normalization control. (D) FACS analysis for quantification of caspase-3 activation in cells transfected with scramble or TRAIL siRNAs and treated with SAHA

3.5 SAHA and MS-275 induce TRAIL protein stabilization

Since our data indicate that SAHA and MS-275 induce TRAIL only at protein level, we hypothesized that HDACis stabilized TRAIL by altering its degradation, likely by proteasome mediation. To demonstrate this hypothesis, we treated both FRTL-5 Cl2 and FRTL-5 *v-ras*-Ki cells with 1 μM or 2.5 μM SAHA in presence or absence of 1 μM MG132, a known proteasome inhibitor. The combined treatment of SAHA with MG132 resulted in a significant increase in TRAIL protein level in transformed cells in comparison with the single treatments. Conversely and in agreement with our previous data (Figure 20A), TRAIL was not detectable in both treated and untreated normal cells. The evaluation of the cell cycle and apoptosis by FACS in normal and transformed thyroid cells in these experimental settings, showed an apoptosis much higher in FRTL-5 *v-ras*-Ki treated with both SAHA and MG132 than that observed for the cells singularly treated (24% with SAHA, 10% with MG132). Conversely, FRTL-5 Cl2 normal thyroid cells were arrested in G1 phase but did not undergo apoptosis after treatment (Figure 20 B and C). These results strongly indicate that SAHA and MG132 synergize in killing tumor cells through the stabilization of death ligand TRAIL.

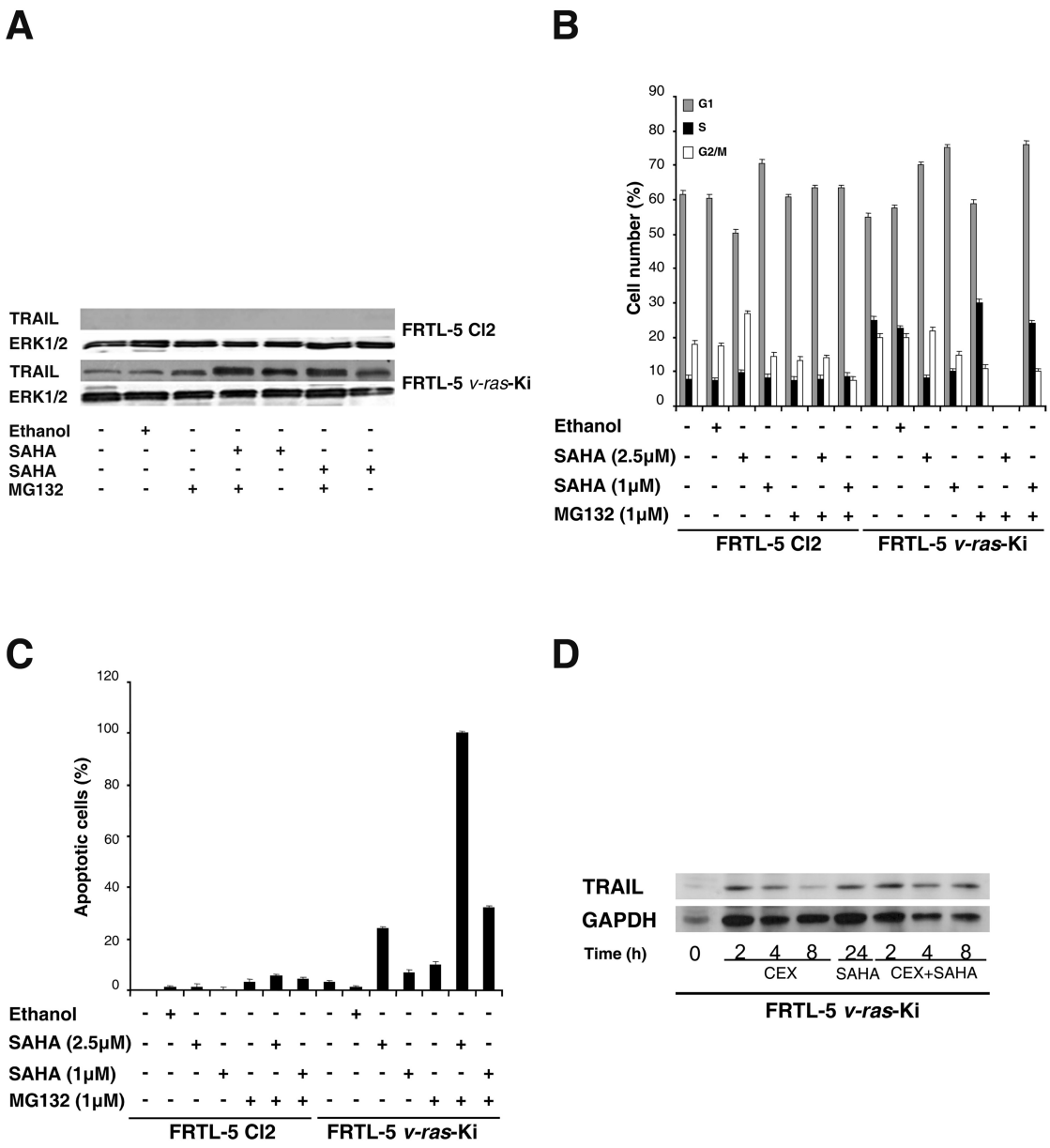


Figure 20. SAHA an MG132 synergize in induction of apoptosis via stabilization of death ligand TRAIL. (A) Western blot analysis of TRAIL in FRTL-5 and FRTL-5 *v-ras*-Ki treated with SAHA, MG132 or the combination SAHA+MG132. Ethanol has been used as vehicle of MG132. ERK1/2 has been used as control for equal protein loading. (B and C) Cell cycle and apoptosis analysis by FACS, of FRTL-5 and FRTL-5 *v-ras*-Ki treated with SAHA,MG132 or the combination SAHA+MG132. (D) Western blot analysis of TRAIL in FRTL-5 *v-ras*-Ki treated with SAHA for 24h and cycloheximide (CEX) for 2, 4 and 8 hours. GAPDH has been used as control for equal protein loading.

In order to confirm that TRAIL is stabilized by HDACis, we analyzed the half-life of TRAIL in FRTL-5 Cl2 and FRTL-5 *v-ras*-Ki treated with 5 μM SAHA for 24 hours and 5 μg/ml cycloheximide for 2, 4 and 8 hours to block mRNA translation. As shown in Figure 20D, TRAIL protein disappeared after 8 hours in FRTL-5 *v-ras*-Ki treated only with cycloheximide, whereas was still present after 8 hours in cycloheximide-SAHA treated cells indicating that SAHA treatment induces an increase of the TRAIL protein half-life.

To further demonstrate that SAHA reduces TRAIL protein degradation affecting the ubiquitin-dependent pathway we co-trasfected FRTL-5 *v-ras*-Ki cells with pEGFP-N1 and p-Ubi-His or with pEGFP-TRAIL and p-Ubi-His. We determined the degradation of TRAIL-GFP fusion protein by evaluating cell fluorescence by FACS after treatment with 5 μM SAHA for 16 hours. The amount of exogenous TRAIL, proportional to the cell fluorescence, increased after treatment with SAHA, whereas was reduced after overexpression of ubiquitin (Figure 21). Taken together, these results indicate that SAHA reduces TRAIL degradation in thyroid transformed cells by altering the ubiquitin-dependent pathway of degradation.

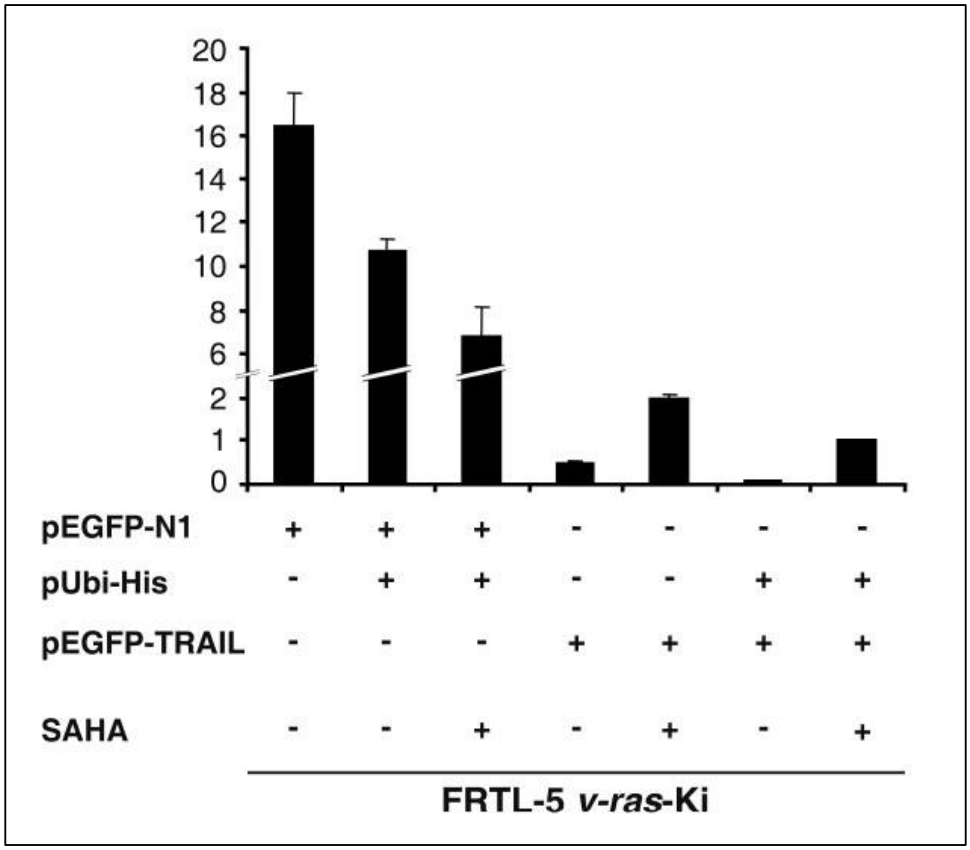


Figure 21. SAHA reduces TRAIL degradation by affecting ubiquitination pathway. FACS analysis to quantify fluorescence and stability of TRAIL-GFP fusion protein in FRTL-5 *v-ras*-Ki cells transfected with pEGFP-TRAIL in presence or absence of p-Ubi-His and treated with SAHA.

3.6 HDACi effects on human anaplastic thyroid carcinoma cell lines

Finally, to demonstrate that our findings can be extended also to human anaplastic cells and can be useful for an alternative therapy of the thyroid anaplastic cancer, we verified the efficacy of the combination MG132+SAHA on FRO, a human anaplastic thyroid cell line. We treated the FRO cells (Fagin J.A.,1993) with 5 μM SAHA, 0.25 μM MG132 or with the combination of both drugs. While the single treatments did not induce cell cycle arrest (Figure 22A), the

combination SAHA + MG132 had a synergistic effect in inducing apoptosis in FRO cells as previously shown for FRTL-5 *v-ras*-Ki cells (Figure 20C). Indeed, the combined treatment caused an evident increase in apoptosis compared to the single treatments (Figure 22B). Analogous results were obtained for FB1 (Figure 22D), another human anaplastic thyroid cell line (Fiore L, 1997), thus suggesting that this combination treatment might be useful against anaplastic thyroid cancer.

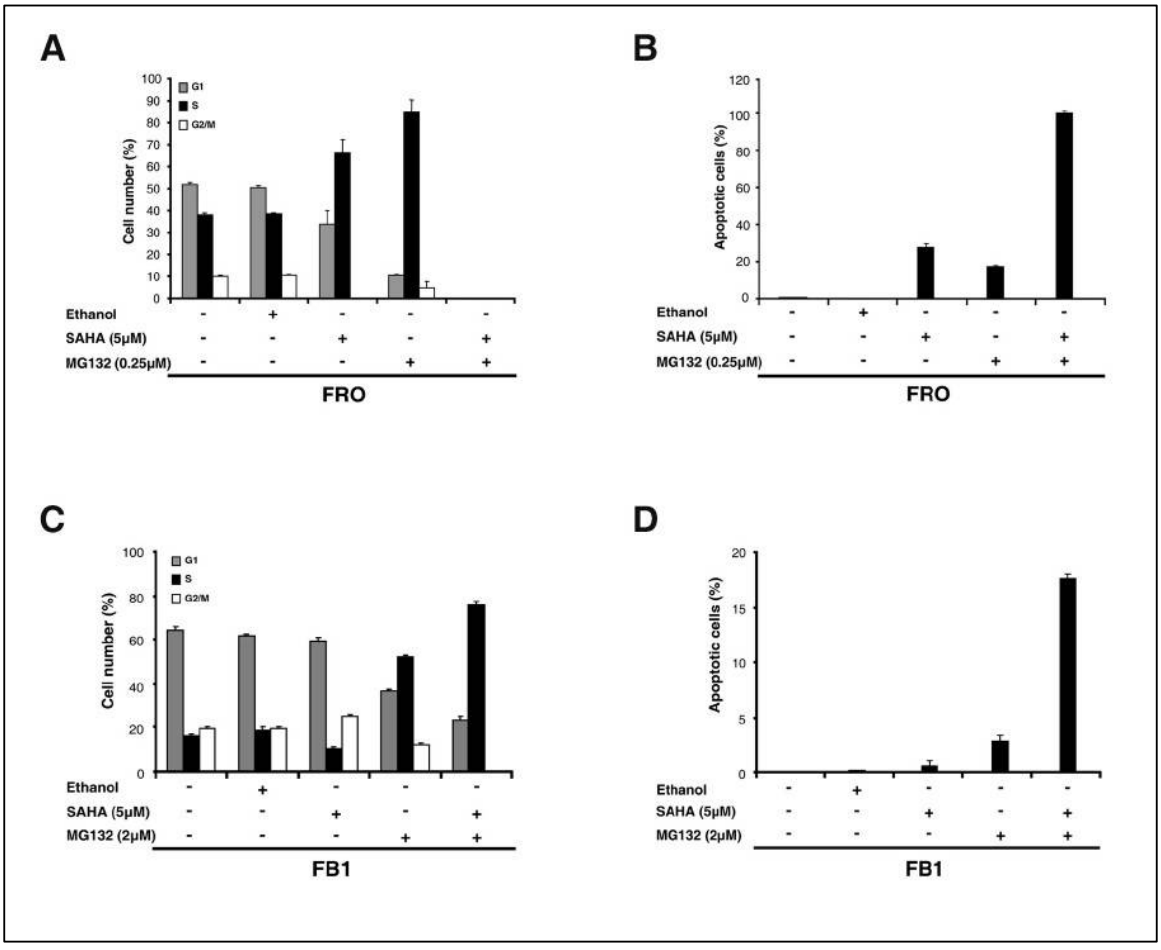


Figure 22. SAHA and MG132 synergize in killing human anaplastic thyroid carcinoma cell lines FRO and FB1. Cell cycle (A and C) and apoptosis (B and D) analyses by FACS of FRO and FB1 cells treated with SAHA and MG132 or the combination SAHA+MG132.

4 DISCUSSION

The anaplastic thyroid carcinoma represents one of the most lethal human neoplasms, being refractory to conventional treatment as chemo- and radiotherapy. Recently, an innovative tumor therapy based on the use of HDACis has been proposed and already introduced in clinical experimentation. Therefore, the elucidation of their mechanisms of action becomes extremely important (Glaser KB, 2007).

In this study we have analyzed the effects of two potent HDAC inhibitors, named SAHA (Vorinostat, Zolinza) and MS-275, in a well defined cell system where the process of tumor transformation is well known allowing a better comprehension of the mechanisms through which these drugs may act. The cell system studied here is represented by the rat thyroid cells infected with the Kirsten murine sarcoma virus carrying the v-*ras*-Ki oncogene (Fusco A,1987). These cells have lost the expression of the typical markers of thyroid differentiation, grow with a high efficiency in soft agar and induce tumors after injection into athymic mice. The critical role of *ras* gene activation in some thyroid cancer histotypes was confirmed by the induction of thyroid follicular carcinomas associated to lung metastasis following the injection of the Kirsten murine sarcoma

virus into the thyroid gland of adult Fischer rats (Portella G, 1989). The importance of *ras* oncogene in thyroid cancer is also evident in human follicular and anaplastic carcinoma in which there is a high frequency of mutation in this gene as described above (Nikiforova, MN, 2003).

SAHA and MS-275 have a different spectrum of action, as SAHA inhibits both class I and II HDACs, whereas MS-275 inhibits only HDAC 1, 2, and 3 to a lower extent. They are already object of clinical trials for the treatment of solid and hematological tumors with minor side effects (Mai A, 2008). Particularly, SAHA (Vorinostat, Zolinza) has been approved by the Food and Drug Administration for the treatment of cutaneous manifestations of T-cell lymphoma in cancer patients. Previous studies have shown that SAHA arrests cell growth, induces apoptosis of a wide variety of transformed cells and inhibits tumor growth in animal models bearing solid tumors and hematological malignancies (Marks PA, 2007). Indeed, SAHA and MS-275 display antitumor activity in acute myeloid leukemia mediated by both p21/WAF1 and the tumor-selective death ligand TRAIL (Nebbioso A, 2005; Insinga A, 2005; Altucci L, 2005). Moreover, the relevance for the mitochondrial pathway of apoptosis after treatment with HDAC inhibitors has been also demonstrated in B cell lymphoma (Lindemann, RK, 2007), thus indicating that more than one mechanism might underlie the HDACi anticancer action

and that tissue context may play a relevant role, and needs to be considered.

In this study, we show that both SAHA and MS-275 induce apoptosis in rat thyroid transformed cells with highly malignant phenotype, but not in normal cells. The increase in the activities of caspase-8 and -9, induced by the treatment with these drugs, suggested that both SAHA and MS-275 can activate mitochondrial and death receptor apoptotic pathways. Despite both BclxL and TRAIL were regulated differentially in normal and tumor cells, we obtained a decrease in HDACi-mediated apoptosis by the silencing TRAIL expression, thus indicating that TRAIL is a key mediator of HDACi-induced apoptosis in thyroid carcinoma cells. The absence of significant differences in TRAIL transcript levels in both normal and transformed cells suggested TRAIL regulation at the protein level. This hypothesis appears confirmed by i) the synergistic apoptotic effect of SAHA and the proteasome inhibitor MG132; ii) the proportional increase of TRAIL protein level in the combo-treatment with respect to the single one; iii) the clear increase in TRAIL half-life induced by SAHA.

Therefore, our results suggest a new molecular mechanism by which HDACis regulate cell death *via* a proteasome-dependent stabilization of TRAIL. Our demonstration that i) TRAIL is degraded through a ubiquitin-proteasome pathway and ii) SAHA stabilizes

TRAIL protein by reducing its proteasome degradation is a fully novel finding that explains, at the molecular level, why the combination of a proteasome and a HDAC inhibitor might be successful in anaplastic thyroid cancers.

In agreement with our findings, the synergism of proteasome inhibitors with HDACis has recently been reported in glioma, in cervical cancer and in leukemia (Miller CP, 2007; Dai Y,2008; Yu, 2008; Miller CP, 2009; Lin Z, 2009) strongly supporting our molecular model and suggesting the application of this combined treatment in other types of cancers.

Thus, the synergism between MG132 and SAHA through TRAIL signaling pathway activation encourages the pre-clinical application of the combination of HDACis with proteasome inhibitors such as Bortezomib, or with drugs able to activate TRAIL apoptotic pathway. Our findings may contribute to the development of a novel, molecularly-based, approach in thyroid cancer treatment.

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