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HDAC INHIBITORS TARGET TRANSCRIPTION FACTORS DEREGULATED IN T-ACUTE LYMPHOBLASTIC LEUKAEMIA

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INDEX

ABSTRACT

Histone deacetylases (HDACs) are enzymes involved in the remodeling of chromatin. In recent years, inhibition of HDACs has emerged as a potential strategy to reverse aberrant epigenetic changes associated with cancer. In fact, HDAC inhibitors (HDACi) promote apoptosis, induce cell cycle arrest and differentiation of tumor cells, by mechanisms which remain in part unknown. T-cell acute lymphoblastic leukemia (T-ALL) is a pediatric malignancy characterized by clonal expansion of lymphoid progenitors. Although the majority of pediatric T-ALL patients can be cured by current protocols, about one fourth of patients has chemotherapy-resistant disease or relapse after therapy and novel therapeutic approaches are required. In our study, we analyzed the effects of HDACi on seven transcription factors important in T-ALL pathogenesis (NOTCH1, NOTCH3, c-MYB, TAL1, TLX1, TLX3 and LMO2) using both established T-ALL cell lines and patient-derived T-ALL xenografts previously obtained in our laboratory. In particular, we focused on transcription factors that define specific T-ALL subgroups (TAL/LMO, TLX1, TLX3) and we included members of the Notch family (NOTCH1 and NOTCH3) and c-MYB in view of their transversal role in T-ALL. *In vitro* analysis highlighted transcriptional down-regulation of C-MYB and TAL1, a post-translation regulation of NOTCH1 and NOTCH3 and the regulation of the transcriptional activity of TLX1 and TLX3 following HDAC inhibition. These biochemical effects were linked to increased apoptosis and impaired proliferation both in T-ALL cell lines and patients-derived cells, partially dependent on NOTCH1 and NOTCH3. We next investigated the *in vivo* effects of the HDACi Givinostat in T-ALL xenografts belonging to specific T-ALL subgroups. Interestingly, PD-TALL8 (TLX1) and PD-TALL16 (TLX3) had better response to treatment compared to PD-TALL12 and PD-TALL9 (TAL/LMO). In fact, Givinostat dramatically decreased leukemic cells infiltrating the spleen and the bone marrow in TLX-driven xenografts, whereas this drug had modest or minimal effects on TAL/LMO xenografts. Taken together, these results identify TLX1 and TLX3 T-ALL subgroups as potential candidates for therapeutic treatment with HDACi such as Givinostat.

RIASSUNTO

Le Istone Deacetilasi (HDACs) sono enzimi coinvolti nel rimodellamento della cromatina. Negli ultimi anni è emerso come l'inibizione delle HDACs potrebbe essere utilizzata come strategia per ripristinare l'alterata regolazione epigenetica che si riscontra nei tumori. Infatti, gli inibitori delle HDAC (HDACi) inducono apoptosi, arresto del ciclo cellulare e differenziamento delle cellule tumorali, ma i meccanismi molecolari alla base di questi fenomeni rimangono poco chiari. La leucemia linfoblastica acuta a cellule T (T-ALL) è un tumore pediatrico caratterizzato dall'espansione clonale di progenitori linfoidi. Nonostante la maggioranza dei pazienti pediatrici affetti da T-ALL siano curati in modo efficace utilizzando gli attuali protocolli terapeutici, circa un quarto dei pazienti manifesta resistenza alla terapia o presenta ricadute e dunque emerge la necessità di nuovi approcci terapeutici. In questo studio abbiamo analizzato gli effetti degli HDACi nei confronti di sette fattori di trascrizione implicati nella patogenesi della T-ALL (NOTCH1, NOTCH3, c-MYB, TAL1, TLX1, TLX3 and LMO2) utilizzando sia linee cellulari stabilizzate, sia modelli murini di T-ALL precedentemente sviluppati nel nostro laboratorio a partire da cellule di pazienti. In particolare, ci siamo concentrati su fattori trascrizionali che identificano specifici sottogruppi di T-ALL (TAL/LMO, TLX1 e TLX3) e abbiamo incluso nell'analisi due membri della famiglia dei recettori Notch (NOTCH1 and NOTCH3) e c-MYB in virtù del loro ruolo oncogenico in questa patologia. Le analisi *in vitro* hanno evidenziato diversi meccanismi di regolazione dei vari fattori da parte degli HDACi. TAL1 e c-MYB risultano regolati a livello trascrizionale, NOTCH1 e NOTCH3 presentano una regolazione post-traduzionale e, nel caso di TLX 1 e TLX3, è presente una regolazione diretta della loro capacità trascrizionale. Gli effetti a livello di proteina si legano all'induzione di apoptosi e all'inibizione della proliferazione sia nelle linee cellulari, sia nelle cellule derivate da paziente e risultano essere parzialmente dovute alla down-modulazione di NOTCH1 e NOTCH3. In seguito siamo andati ad indagare la risposta *in vivo* dell'HDACi Givinostat in xenografts di T-ALL appartenenti a specifici sottogruppi genetici. E' interessante notare che il trattamento ha avuto il maggiore risultato nelle PD-TALL8 (TLX1) e nelle PD-TALL16 (TLX3) rispetto alle PD-TALL12 e le PD-

TALL9 (entrambe TAL/LMO). Infatti, il trattamento con Givinostat negli xenografts di tipo TLX determina una riduzione dell'infiltrazione da parte delle cellule leucemiche nella milza e nel midollo mentre gli effetti ottenuti negli xenografts TAL/LMO risultano modesti o addirittura nulli. In conclusione, i dati ottenuti identificano i pazienti di T-ALL appartenenti ai sottogruppi TLX1 e TLX3 come potenziali candidati per il trattamento a scopo terapeutico con HDACi come il Givivnostat.

1. INTRODUCTION

1.1 T-CELL ACUTE LYMPHOBLASTIC LEUKAEMIA

1.1.1 Pathogenesis

T-cell acute lymphoblastic leukaemia (T-ALL) is a neoplastic disorder of the lymphoblast committed to the T-cell lineage. T-ALL represents 15% of childhood and 25% of adult ALL and is characterized by an unfavourable prognosis compared to B cell ALL. Patients present high levels of circulating blasts, infiltration of bone marrow and spleen and, in some cases, involvement of central nervous system (Graux et al., 2006). T-ALL is thought to result from malignant thymocytes that arise at defined stages of intrathymic T-cell differentiation. Transformation events occur in crucial steps of thymocyte development and determine an arrest at particular stages of normal thymocyte development (Aifantis et al., 2008). Sequential alterations in proto-oncogenes, tumour-suppressor genes, and microRNA genes could involve hematopoietic stem cells or their committed progenitors. These fundamental changes alter key regulatory processes in target cells by conferring an unlimited capacity for self-renewal, altering the controls of normal proliferation, blocking cell differentiation, and promoting resistance to death signals (Pui, 2009).

Cytogenetic analysis of lymphoblasts reveals recurrent translocations that activate a small number of oncogenes in 25–50% of T-ALL but a large proportion of T-ALL shows a normal karyotype. The abnormal karyotypes found in approximately 50% of T-ALL cases (Table 1), however, are still less than the percentage found in B-cell-lineage ALL cases (Aifantis et al., 2008).

Cytogenetic abnormality	Approximate frequency	Involved oncogenes or fusion genes
Translocations involving TCR genes on chromosomes 7q34 (*TCRB* and *TCRG*) and 14q11 (*TCRA* and *TCRD*)	35%	*HOX11*, *HOX11L2*, *TAL1*, *TAL2*, *LYL1*, *BHLHB1*, *LMO1*, *LMO2*, *LCK*, *NOTCH1*, cyclin D2
Abnormal expression	9–30%	*SIL–TAL1* fusion
Fusion gene formation	10%	*CALM–AF10* fusion
	4–8%	*MLL* fusions
	6%	*ABL1* fusions
	Rare	*NUP98* fusions
Chromosomal deletions of 9p21 and 6q	Up to 65% (9p21)	*P15*, *P16*
	20–30% (6q)	Unknown
Activating gene mutations	50–60%	*NOTCH1*, *FLT3*, *NRAS*
Gene duplications	~30% in T-ALL cell lines	*MYB*

Table 1: Common cytogenetic abnormalities in T-ALL (Aifantis et al., 2008).

The most frequent genetic abnormalities can be divided in:

- *Translocations involving the T cell receptor (TCR).* About 40% of T-ALL harbour chromosomal translocations juxtaposing a transcription factor, important in thymocytes development, next to strong regulatory elements (promoter and enhancer) located near to the T-cell receptor β (TCRB) gene on chromosome 7 or the T-cell receptor α-δ (TCRAD) locus on chromosome 14. These chromosomal translocations can occur during thymocyte development as results of uncorrected TCR recombination and lead to aberrant gene expression giving rise to T cells that show abnormal cell cycle control, proliferation and differentiation. The most frequent translocations involve HOX genes (including HOX11, HOX11L2, also known as TLX1 and TLX3 and HOXA) or genes coding for proteins interacting with E2A (TAL1, TAL2, LYL1, BHLHB1, LMO1 and LMO2) (Tosello and Ferrando, 2013).
- *Formation of chimeric protein with oncogenic properties.* Additional genetic abnormalities in T cells from patients with T-ALL include chromosomal translocations that generate fusion genes encoding new chimeric proteins with oncogenic properties, such as the SIL-TAL1 fusion protein and fusion proteins that involve MLL, ABL1 and NUP98.
- *Tumour suppressor deletions.* The most common cryptic deletions in T-ALL, leading to the loss of tumour suppressors, are deletions of the INK4 (also known as CDKN2A) locus at chromosome 9p21, which contains genes encoding the cyclin-dependent kinase inhibitor p16 and other proteins important in regulation of the cell cycle (Aifantis et al., 2008).
- *Activating mutations.* Recently, it has also been found that more than 50% of T-ALL cases has activating mutations in the key regulator of T-cell fate NOTCH1 (Weng et al., 2004). These activating mutations lead to high levels of NOTCH1 signalling and give a proliferation advantage to the cell, mainly due to secondary effects on key regulators of cell cycle and apoptosis (Palomero et al., 2006). Moreover, 15% of T-ALL patients harbour mutations or deletions in the ubiquitin-ligase FBW7, responsible for NOTCH1 ICD degradation, mimicking the effects of Notch1 mutations

(O'Neil et al., 2004). Other oncogenes that can present activating mutations are NRAS and FLT3.

- *Duplications.* Recent studies have explored the importance of the transcription factor c-MYB in T-ALL. In particular, a duplication of the transcription factor c-MYB was identified in about 8% of T-ALL patients (Clappier et al., 2007; Lahortiga et al., 2007) and in about 30% of T-ALL cell lines (Aifantis et al., 2008).

Moreover, altered expression or ectopic activation of transcription factors in T-ALL are reported, without evident cytogenetic alterations. Regarding this, it was previously reported that also enforced expression of Notch3-ICD is a potent inducer of T cell leukaemia in mouse model and that Notch3 over-expression characterizes human T-ALL, even if mutations in this Notch paralog have not been reported (Bellavia et al., 2000; Screpanti et al., 2003).

1.1.2 Gene expression signatures identify different T-ALL subgroups

Careful analysis of clonal chromosomal abnormalities in leukemic blast cells had a greater impact on the B lineage leukaemias than on T cell acute lymphoblastic leukaemia (T-ALL), whose pathogenesis and molecular subtypes remained, for long time, largely undefined. In fact, the different clinical outcome of T-ALL patients reflects a molecular heterogeneity that cannot be appreciated using conventional cytogenetic analysis. Gene expression analysis using oligonucleotide or cDNA microarrays have been used by several groups as a novel tool for delineating molecular pathways that drive the malignant transformation of developing thymocytes (Ferrando et al., 2002; Ross et al., 2003; Soulier et al., 2005; Yeoh et al., 2002). Some genetic abnormalities occur in a mutually exclusive manner and have been defined as type A mutations. Type A mutations involved driving oncogenes and, based on gene expression profiling, define four main distinct genetic T-ALL subgroups: TAL1/LMO, TLX1, TLX3 and HOXA (Table 2).

Subgroup	Rearrangement	Gene(s)	T-cell arrest[a]	Outcome	Frequency (%)
TAL/LMO	t(1;14)(p32;q11)/ t(1;7)(p32;q34)/ 1p32 deletion	*TAL1*	pre-αβ/TCR-αβ [3,6,12,15,17,83,84]	Good [9,12,14,85]	4 [6,12–14,16] 15 [6,12–14,16]
	t(7;9)(q34;q32)	*TAL2*	Unknown	Unknown	1–2 [5]
	t(11;14)(p15;q11)/ t(7;11)(q34;p15)	*LMO1*	Unknown	Unknown	1–2 [5]
	t(11;14)(p13;q11)/ t(7;11)(q34;p13)/ 11p13 deletion	*LMO2*	immature/pre-αβ/TCR- αβ [17]	Unknown	6 [17,18] 3 [17,18]
	t(7;12)(q34;p12)	*LMO3*	Unknown	Unknown	< 1 [22]
TLX1	t(10;14)(q24;q11)/ t(7;10)(q34;q24)	*TLX1/HOX11*	immature/pre-αβ [6,12,17,78,79,83]	Good [9,80–82]	8 [9,12]
TLX3	t(5;14)(q35;q32)[b]	*TLX3/HOX11L2*	immature/pre-αβ/γδ lineage [6,12,17]	Poor [3,12,86,87] No impact [9,88] Good [97]	24 [6–12]
HOXA	inv(7)p15q34)/ t(7;7)(p15;q34)	*HOXA*	TCR-αβ or TCR-γδ [98]	Undefined	3 [4,98,100]
	t(10;11)(p13;q14)	*CALM-AF10*	immature/γδ lineage [12,99]	Poor [12,99]	
	t(11;19)(q23;p13)[c]	*MLL-ENL*	immature/γδ lineage [29]	Unknown	2–5 [7,12,99]
	9q34 deletion	*SET-NUP214*	immature/γδ lineage [5]	Unknown	< 1 [29] 3 [5]
MYB	t(6;7)(q23;q34)	*MYB*	Unknown	Unknown	3 [101]
ETP	High *LYL1* expression	*unknown*	immature	Poor [3,75]	10 [3,4,75]

Table 2: Genetic subgroups in paediatric T-cell acute lymphoblastic leukaemia (Meijerink, 2010).

The TLX3 and TLX1 subgroups are exclusively characterized by rearrangements of the TLX3 and TLX1 oncogenes respectively. TLX1 and TLX3 are over-expressed in 10% and 30% of paediatric T-ALL, respectively, as a consequence of chromosomal translocations. Genes bound by these homeobox transcription factors are characteristically down-regulated, suggesting that TLX1 and TLX3 primarily function as transcriptional repressors. In particular, in neural stem cells it has been demonstrated that TLX1 and TLX3 recruit histone deacetylases (HDACs) and form a silencing complex on target genes promoter (Sun et al., 2007). TLX1 and TLX3 are implied in T-cell maturation and, in particular, they act as antagonists of physiological thymocyte differentiation. In this regard, TLX1/3 silencing in human T-ALL cell lines increases differentiation and cell death, suggesting a differentiation arrest and induction of apoptosis. Moreover, T-ALL patients harbouring TLX1/3 signature are characterized by early cortical thymocytes, confirming their effect on T-cell differentiation (Dadi et al., 2012). In contrast to TLX1/3 subgroups, the TAL/LMO and HOXA subgroups seem to be characterized by the presence of various rearrangements affecting several, but functionally equivalent oncogenes. TAL1 and LMO2 normally participate in the same transcriptional complex that regulates the activity of the important E2A/HEB transcription factors (O'Neil et al., 2004). This may explain why TAL1 or LMO2-rearranged T-ALL cases have highly similar if not identical gene

expression profiles. Mutations that are found in more than one genetic subgroup are the so-called type B mutations (Table 3). These mutations affect various cellular processes, and include loss of cell cycle inhibitors p15 and p16, activation of the NOTCH1 pathway, activating mutations in the RAS pathway, mutations resulting in the activation of the AKT pathway, or chromosomal rearrangements that result in fusion proteins with tyrosine kinase activity (Meijerink, 2010).

	Rearrangement	Gene(s)	Function	Outcome	Frequency (%)
Cell cycle defects	9p21 deletion/ hypermethylation	*CDKN2A/2B*	Cell cycle inhibitor/Inhibitor HDM2	Unknown	70 [33,34]
	t(7;12)(q34;p13)/ t(12;14)(p13;q11)	*CCND2*	Cell cycle activator	Unknown	< 1 [102]
NOTCH pathway	t(7;9)(q34;q34)	*NOTCH1*	Self-renewal, differentiation T-cell commitment	GPR [49,50,53] Reduced MRD [49,50] Good [49,51,54,103]	< 1 [57,107]
	Activating mutation[a]	*NOTCH1*		Poor [53,104]	> 60 [37,56]
	Inactivating mutation	*FBXW7*	Proteasomal degradation	no impact [17,50,52–54,105,106]	8–30 [35,36,55]
AKT pathway	10q23.31 deletion/ Inactivating mutation	*PTEN*	Signal transduction	Poor [39]/no impact [39]	8 [39] 17–27 [39,43]
	Activating mutation	*AKT*	Signal transduction	no impact [39]	2 [39]
	Activating mutation	*PI$_3$K*	Signal transduction	no impact [39]	7 [39]
RAS pathway	Activating mutation	*N/K-RAS*	Signal transduction	Unknown	4–10 [39–42]
	17q11.2 deletion/mutation	*NF1*	RAS inhibitor	Unknown	3 [38,39]
Additional deregulation	Duplication	*MYB*	Transcription factor	Unknown	8–15 [101,109,110]
T-cell differentiation	Inactivating mutations	*PHF6*	Tumor suppressor	No impact [108]	16–38 [108]
Activation other tyrosine kinases	episomal 9q34 amplification	*NUP214-ABL1*	Signal transduction	Poor [44] No impact [111]	4 [44,111]
	t(9;14)(q34;q32)	*EML1-ABL1*	Signal transduction	Unknown	< 1 [113]
	t(9;12)(q34;p13)	*ETV6-ABL1*	Signal transduction	Unknown	< 1 [114]
	t(9;22)(q34;q11)	*BCR-ABL1*	Signal transduction	Poor [45]	< 1 [45]
	t(9;12)(p24;p13)	*ETV6-JAK2*	Signal transduction	Unknown	< 1 [115]
	Activating mutation	*FLT3*	Tyrosine kinase receptor	No impact [112]	2–4 [112,116]

Table 3: Classification of common abnormalities in T-cell acute lymphoblastic leukaemia (Meijerink, 2010).

Common aberrations found in T-ALL are NOTCH1 activating mutations that occur in over 60% of the T-ALL leukaemias. These mutations involve activating mutations in NOTCH1 itself or inactivating mutations in the E3-ubiquitin ligase gene FBXW7. These mutations are found in all genetic subgroups, although the incidence of NOTCH1 activating mutations is relatively higher for TLX3-rearranged cases, but lower within the TAL/LMO subgroup. NOTCH1 mutations affecting the heterodimerization domain (HD) result in a ligand-independent release of the intracellular domain of NOTCH1 (ICD), which subsequently translocates into the nucleus where it acts as a transcription factor. Alternatively, NOTCH1 PEST domain mutations or inactivating FBXW7 mutations preserve ICD from ubiquitin-mediated degradation in the proteasome. Interestingly, although Notch1 is a potent inducer of T-leukaemia and activating mutations are founded in a high percentage of T-ALL patients, this oncogene does not identify a specific subgroup.

1.1.3 Treatment of ALL

Current therapies for ALL can cure more than 80% of children and fewer than 50% of adults with this haematological malignancy. For this reason new therapeutic approaches are required (Pui and Evans, 2006). Children with ALL are usually treated according to risk groups defined by both clinical and laboratory features. The intensity of treatment to achieve favourable outcome varies substantially according to patients stratification. Risk-based treatment assignment is utilized in children with ALL in order to spare unnecessary intensive and toxic treatment. Therapy of T-ALL is generally divided into three phases: (I) remission/induction therapy followed by (II) consolidation/intensification therapy and, finally, (III) maintenance/continuation treatment. Certain ALL study groups, such as the Children's Oncology Group (COG), use a more or less intensive induction regimen based on pre-treatment factors, while other groups give a similar induction regimen to all patients. Factors used by the COG to determine the intensity of induction include immunophenotype and the National Cancer Institute (NCI) risk group classification. The NCI risk group classification stratifies risk according to age and white blood cell (WBC) count:

- **Standard risk** - WBC count less than 50,000/μL **and** age 1 to younger than 10 years.
- **High risk** - WBC count 50,000/μL or greater **and/or** age 10 years or older.

In addition to this classification, ALL patients can be stratified according to other prognostic factors:

- **Patients characteristics.** Central Nervous System (CNS) and testicular involvement at diagnosis are adverse prognostic factors. Regarding gender, the prognosis for girls with ALL is slightly better than it is for boys with ALL. This could be explain by the occurrence of testicular relapses among boys, but boys also appear to be at increased risk of bone marrow and CNS relapse for reasons that are not well understood. Down Syndrome and race are also considered prognostic factors in induction treatment.

- **Leukemic cells characteristics.** Leukemic cell characteristics affecting prognosis include morphology, immunophenotype (B-ALL or T-ALL) and cytogenetic/genomic alterations. Recurrent chromosomal abnormalities have been shown to have prognostic significance, especially in B-precursor ALL.

- **Response to initial treatment.** Long-term outcome is associated with elimination of leukemic cells after initial treatment and the level of residual disease at the end of induction phase. Treatment response is influenced by the drug sensitivity of leukemic cells and host pharmacodynamics and pharmacogenomics. For this reason, early response has strong prognostic significance. Leukaemia response is evaluated by minimal residual disease (MRD) parameters that allow to monitored leukemic cells in the bone marrow using molecular features that are not expressed by normal lympho-hematopoietic cells (clonal rearrangement of immunoglobulin (IG) and T-cell receptor (TCR) genes and chromosomal abnormalities by PCR, cell marker profiles by flow cytometric analysis). (Campana and Coustan-Smith, 2012) and http://www.cancer.gov/cancertopics/pdq/treatment/adultALL/HealthProfessional, National Cancer Institute web site).

Standard remission-induction therapy for newly diagnosed childhood ALL include a multidrug chemotherapy with vincristine, corticosteroid (prednisone or dexamethasone), L-asparaginase and an anthracycline (doxorubicin or daunorubicin). Glucocorticoids were among the first drugs used in the treatment of ALL and have remained essential components of therapy. Their cytotoxic effect seems to be mediated through binding of glucocorticoid receptors and consequent inhibition of cytokine production, alteration of the expression of various oncogenes, and induction of cell cycle arrest and apoptosis. Glucocorticoid resistance is an adverse prognostic factor in ALL, and several mechanisms have been reported (Inaba and Pui, 2010). The goal of remission-induction therapy is to eradicate more than 99% of the initial burden of leukaemia cells and to restore normal haematopoiesis (Pui and Evans, 2006). The vast majority of children with ALL achieve complete morphologic remission by the end of the first month of treatment. However, the presence of more than 5% lymphoblasts at the end of the induction phase is observed in up to 5% of children with ALL. Once complete remission (CR) has been achieved, systemic

treatment in conjunction with CNS-directed therapy follows. Standard treatment options for CNS-directed therapy include intratecal chemotherapy (usually methotrexate), CNS-directed Systemic Chemotherapy and, in some cases, cranial radiation. The intensity of the post-induction chemotherapy varies considerably depending on risk group assignment, but all patients receive some form of intensification after the achievement of CR and before beginning maintenance therapy. Commonly used regimens for child-hood ALL include high-dose methotrexate with mercaptopurine, high-dose asparaginase given for an extended period, and reinduction treatment. Finally, patients are subjected to maintenance/continuation treatment in order to eliminate residual leukaemia cells. Patients with ALL require prolonged continuation therapy, at least two years, and attempts to shorten this phase have yielded poor results in both children and adults. The maintenance/continuation therapy is based on the combination of methotrexate and mercaptopurine administrated weekly and daily respectively, with or without pulses of dexamethasone and vincristine (Pui and Evans, 2006).

1.2 HISTONE DEACETYLASES (HDACs)

1.2.1 HDACs family: structure and functions

Transcription in eukaryotic cells is influenced by DNA organization in the nucleus. In fact, DNA is packaged into chromatin, a highly organized and dynamic protein-DNA complex. The core subunit of chromatin is the nucleosome, composed of an octamer of four core histones, an H3/H4 tetramer and two H2A/H2B dimers, surrounded by 146 bp of DNA. Local chromatin organization is generally recognized as an important factor in the regulation of gene expression. During activation of gene transcription, the inaccessible structure of DNA become available to DNA binding proteins through modification of the nucleosomes. Remodelling of chromatin is strongly influenced by post-translational modifications of the histones, such as acetylation, methylation, phosphorylation, poly-ADP ribosylation, ubiquitinylation, sumoylation, carbonylation and glycosylation. Compared with other modifications, acetylation of core histones is probably the best understood type of epigenetic modification. The balance between "open" and "close" chromatin forms is driven by two classes of enzyme with different activities: histone acetyltransferases (HATs) transfer acetyl groups to amino-terminal lysine residues of histones, which results in local expansion of chromatin and increased accessibility of regulatory proteins to DNA, whereas histone deacetylases (HDACs) catalyze the removal of acetyl groups, leading to chromatin condensation and transcriptional repression (Fig.1) (de Ruijter et al., 2003).

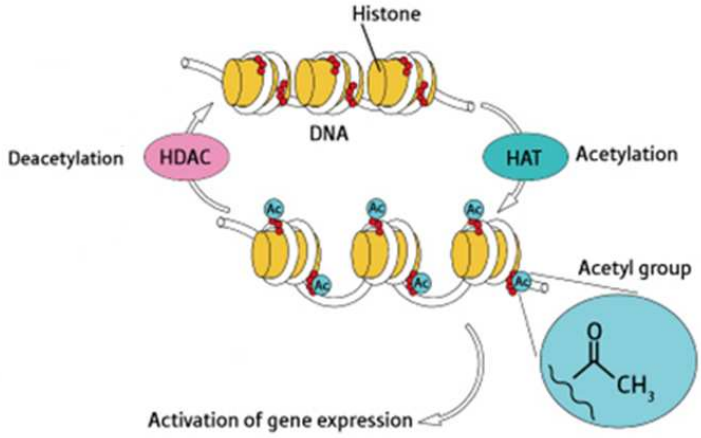


Fig.1: HDACs and HATs opposite activities on chromatin condensation in the nucleus.

Eighteen HDACs have been identified in humans, and they are subdivided into two protein families: the Zn^{2+} dependent classical HDAC family (class I, II and IV), and the Zn^{2+}-independent NAD+-dependent Sirtuins (class III). The classical HDAC family consists of four classes based on their homology to yeast HDACs, their subcellular localization and their enzymatic activities. The class I HDACs (1, 2, 3 and 8) are homologous to the yeast RPD3 protein, can generally be localized in the nucleus and are ubiquitously expressed in various human cell lines and tissues. Class II HDACs (4, 5 ,6, 7, 9 and 10) share homologies with the yeast Hda1 protein and can shuttle between the nucleus and the cytoplasm. This subgroup is further divide in class IIa (4, 5, 7 and 9) and class IIb (6 and 10). In particular, HDACs 6 and 10 are found in the cytoplasm and contain two deacetylase domains. HDAC6 has unique substrate specificity with an α-tubulin deacetylase (TDAC) domain specific for the cytoskeletal protein α-tubulin. HDAC11 is the only member of the class IV HDACs. It shares sequence similarity with the catalytic core regions of both class I and II enzymes but does not have strong enough identity to be placed in either class. The class III HDACs (SIRT1, 2, 3, 4, 5, 6 and 7) are homologues of the yeast protein Sir2 and require NAD+ for their activity to regulate gene expression in response to changes in the cellular redox status. (Table 4)(Bolden et al., 2006; Thiagalingam et al., 2003).

Classification			Location	Function
Zn^{2+}-dependent	Class I	HDAC1	Nucleus	Participate in Sin3, NuRD (nucleosome remodeling and deacetylation) and Co-REST complex
		HDAC2	Nucleus	
		HDAC3	Nucleus, rarely in cytoplasm	Participate in SMRT (silencing mediator for retinoic acid and thyroid hormone receptors), N-CoR (nuclear receptor co-repressor) complex
		HDAC8	Nucleus	-
	Class IIa	HDAC4	Nucleus, cytoplasm	Interaction with SMRT/N-CoR and the co-repressors BcoR (Bcl-6-interacting co-repressor) and CtBP
		HDAC5	Nucleus, cytoplasm	
		HDAC7	Nucleus, cytoplasm	
		HDAC9	Nucleus, cytoplasm	Muscle differentiation
	Class IIb	HDAC6	Cytoplasm	Tubulin deacetylase
		HDAC10	Nucleus, cytoplasm	Recruitment other HDACs
	Class IV	HDAC11	Nucleus, cytoplasm	-
Zn^{2+}-independent	Class III	SIRT1-7		

Table 4: The classification of HDACs in mammals (Pan et al., 2007).

1.2.2 Non-histone proteins as substrates of HDACs

Interestingly, despite the importance of HDACs in the regulation of genes expression, genome-wide transcript profiling by microarrays has indicated that a relatively small percentage of genes (between 2% and 5%) is influenced by HDAC inhibition (Glaser et al., 2003; Johnstone and Licht, 2003). To this end, recent studies focused their attention on HDACs target alternative to histones. Recent phylogenetic analyses of bacterial HDACs suggest that all four HDAC classes preceded the evolution of histone proteins (Gregoretti et al., 2004). This suggests that the primary activity of HDACs may be directed against non-histone substrates. At least 50 non-histone proteins of known biological function have been identified, which may be acetylated and substrates of HDACs. Non-histone protein targets of HDACs include transcription factors, transcription regulators, signal transduction mediators, DNA repair enzymes, nuclear import regulators, chaperone proteins, structural proteins, inflammation mediators and viral proteins (Table 5) (Xu et al., 2007).

Function	*Proteins*
DNA binding transcriptional factors	p53, c-Myc, AML1, BCL-6, E2F1, E2F2, E2F3, GATA-1, GATA-2, GATA-3, GATA-4, Ying Yang 1 (YY1), NF-κB (RalA/p65), MEF2, CREB, HIF-1α, BETA2, POP-1, IRF-2, IRF-7, SRY, EKLF
Steroid receptors	Androgen receptor, estrogen receptor α, glucocorticoid receptor
Transcription coregulators	Rb, DEK, MSL-3, HMGI(Y)/HMGA1, CtBP2, PGC-1α
Signaling mediators	STAT3, Smad7, β-catenin, IRS-1
DNA repair enzymes	Ku70, WRN, TDG, NEIL2, FEN1
Nuclear import	Rch1, importin-α7
Chaperone protein	HSP90
Structural protein	α-Tubulin
Inflammation mediator	HMGB1
Viral proteins	E1A, L-HDAg, S-HDAg, T antigen, HIV Tat

Table 5: Non-histone substrates of HDACs (Xu et al., 2007).

Unlike other histone deacetylases with chromatin remodelling activity, HDAC6 catalyzes deacetylation of cytoplasmic substrates, such as α-tubulin, Hsp90, and cortactin. In particular, it has been demonstrated that HDAC6 inhibition determines increased acetylation of α-tubulin (Zhang et al., 2003) and, consequently, promotes the association of microtubules with dynein and kinesin motors, leading to increased motor processivity and secretory vesicle flux. These

results show a potentially regulatory role for HDAC6 in endocytic cargo transport (Gao et al., 2010; Reed et al., 2006). Moreover, this HDAC is implied in the aggresome formation, an aggregate of ubiquitinated misfolded proteins, commonly found in neurodegenerative pathologies and tumour cells (Boyault et al., 2007; Rodriguez-Gonzalez et al., 2008).

1.2.3 HDACs as targets in cancer treatment

The fact that acetylation is a key component in the regulation of gene expression has stimulated the study of HDACs in relation to the aberrant gene expression often observed in cancer. Cancer has traditionally been considered a disease caused by genetic alterations such as gene mutations, deletions and chromosomal abnormalities, that result in the loss of function of tumour-suppressor genes and/or gain of function or hyper-activation of oncogenes. However, there is growing evidence that gene expression regulated by epigenetic changes is also important for the progression of cancer. Recent studies have focused the attention on the aberrant recruitment of HDACs by oncogenic DNA-binding proteins, resulting from chromosomal translocations. For example, the oncogenic PML–RARα, PLZF–RARα and AML1–ETO fusion proteins induce acute promyelocytic leukaemia(APL) and acute myeloid leukaemia (AML) by recruiting HDAC-containing complexes that constitutively repress the expression of specific target genes (Lin et al., 2001). In addition to aberrant recruitment of HDACs to specific loci, altered expression of individual HDACs in tumours has also been reported. HDAC1 is over-expressed in prostate (Halkidou et al., 2004), gastric (Choi et al., 2001), colon, breast carcinomas (Zhang et al., 2005). HDAC2 showed higher expression in colorectal (Zhu et al., 2004), cervical and gastric cancer (Song et al., 2005). Increased expression of HDAC3 is seen in colon tumours (Wilson et al., 2006). HDAC6 over-expression is observed in breast cancer (Zhang et al., 2004). Concerning haematological tumours, few works examined the differentially expressed HDACs in ALL patients. In 2010 Moreno and colleagues (Moreno et al., 2010) identified HDAC3, HDAC7 and HDAC9 as the most over-expressed HDACs in ALL patients and their altered expression was associated with poor prognosis in childhood ALL. Similar results were obtained by Gruhn et al. in

2013 (Gruhn et al., 2013). In addition to the previous work, a correlation analysis of HDAC expression with clinico-pathological parameters revealed that high HDAC4 expression was associated with prednisone poor-response in T-ALL patients. Moreover, siRNA-mediated inhibition of HDAC4 sensitized a T-ALL cell line to etoposide induced cell death.

At present, the relationships between aberrant expression of various HDACs and cancer remain unclear; however, siRNA-mediated knockdown of HDACs over-expressed in different cancer cell lines decreased tumour growth and improved survival. Taken together, these data support the hypotesis that altered expression of HDACs could have an active role in tumour establishment and progression, and highlight HDACs as attractive targets for therapeutic intervention.

1.3 HDAC INHIBITORS (HDACi)

1.3.1 HDACi structure and classification

Epigenetic changes identified in tumours can be genome-wide or more restricted and can alter the expression or activity of a defined epigenetic regulatory protein. These evidences provide a strong rationale for the use of epigenetic-based therapies such as HDACi in cancer. A large number of structurally diverse HDACi have been purified from natural sources or synthetically produced, and at least 11 drugs have entered clinical development. HDACi can be classified according to their chemical structure into hydroxamates, cyclic peptides, benzamides and fatty acids (Marks, 2010), or according to their specificity for various HDAC classes.

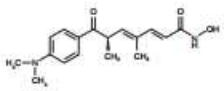
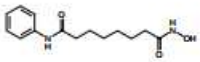
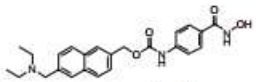
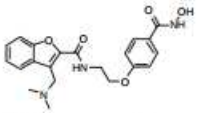
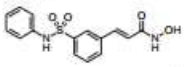
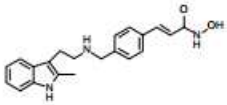
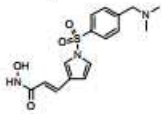
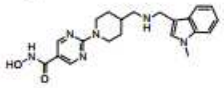
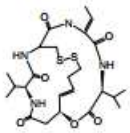
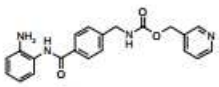
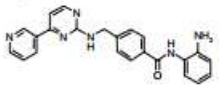
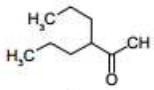
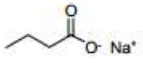
HDAC inhibitor	Structure	HDAC class specificity	Potency	Clinical trials
Hydroxamic acids				
Trichostatin A (TSA)		I, II, IV	nM	–
Vorinostat (suberoylanilide hydroxamic acid, SAHA)		I, II, IV	nM	FDA-approved (2006), phase II, III
Givinostat (ITF2357)		I, II	nM	Phase I, II
Abexinostat (PCI-24781)		I, II, IV	nM	Phase I, II
Belinostat (PXD101)		I, II, IV	μM	Phase I, II
Panobinostat (LBH589)		I, II, IV	μM	Phase II, III
Resminostat (4SC-201)		I, II, IV	μM	Phase I, II
Quisinostat (JNJ-26481585)		I, II, IV	μM	Phase I
Cyclic peptide				
Depsipeptide (romidepsin)		I	nM	FDA-approved (2009), phase I, II
Benzamides				
Entinostat (MS-275)		I	μM	Phase II
Mocetinostat (MGCD0103)		HDAC1	μM	Phase I, II
Fatty acids				
Valproic acid (VPA)		I, II	mM	Phase I, II, III
Butyrate		I, II	mM	Phase II

Table 6: HDACi classification by chemical structure and clinical trial use (Ververis et al., 2013).

The inhibitory effect of a large number of HDAC inhibitors is due to the Zn^{2+} dependency of HDAC enzymes. In fact, many HDACi function by blocking access to the active site of HDAC, in a reversible or irreversible manner. The first HDACi discovered so far is TSA, a fermentation product of Streptomyces.

Originally TSA was used as an anti-fungal agent, but later it was assessed to have potent proliferation-inhibitory properties in cancer cells. Today TSA is used mainly as a reference substance in research for newly developed HDACi (de Ruijter et al., 2003).

1.3.2 Biological effects of HDACi on tumour cells

Treatment with HDACi induces tumour cell death, in particular apoptosis, differentiation and cell cycle arrest *in vitro* and *in vivo*. In addition, activation of the host immune response and inhibition of angiogenesis might also have important roles in HDACi-mediated tumour regression *in vivo.* HDACi selectively induce apoptosis in tumour cells but not in the normal counterpart, implying a therapeutic potential. In fact, normal cells are generally more resistant than tumour cells to the pro-apoptotic effects of HDACi. Clinical trials and preclinical experiments demonstrated that HDACi can have potent anticancer activities at concentrations that are minimally toxic to the host (Bolden et al., 2006; Minucci and Pelicci, 2006).

Although it is well known that HDACi can kill tumour cells, the molecular pathways that are responsible for this effect remain to be fully elucidated. In addition, the drug exposure times and dose for *in vitro* assays can differ markedly from those achievable *in vivo.* Given the pleiotropic biological effects of HDACi, it is clear that the effects of these compounds on a single molecular pathway cannot mediate apoptosis, cell cycle arrest and differentiation in all cell types. The main molecular mediators implied in HDACi effects are listed below.

- **Apoptotic pathways**. HDAC inhibitors have been reported to activate both the death-receptor and intrinsic apoptotic pathways. A large number of studies has demonstrated that various Tumour Necrosis Factors (TNF) receptor super family members and their ligands are transcriptionally activated following HDACi treatment and numerous studies correlate HDACi-related apoptosis with induction of one or more extrinsic death receptors and/or ligands in human leukaemia cells, including T-ALL cell lines Jurkat (Rosato et al., 2003). Moreover, also intrinsic mitocondrial

apoptotic pathway seems to play a role in HDACi apoptosis. In this regard, HDACi-mediated apoptosis in CEM-CCRF T-ALL cell line through intrinsic pathway is due to a selective activation or induction of apoptotic proteins belonging to BH3-only proteins (Bim, Bid and Bmf) (Ruefli et al., 2001). At the same time, HDACi increase levels of Reactive Oxygen Species (ROS) and treatment with free radicals scavengers suppress apoptotic activity of these drugs in multiple myeloma cell lines. In particular, the HDACi SAHA causes an increase in the level of Trx, a major reducing protein, in normal cells but not in transformed cells (Ungerstedt et al., 2005).

- **Cell cycle arrest and differentiation.** Treatment with HDACi is associated with cell-cycle arrest at the G1/S. This is often associated with the p53-independent induction of CDKN1A, which promotes hypophosphorylation of pRb (Sandor et al., 2000). HDACi can also mediate G2/M-phase arrest by activating a G2-phase checkpoint, although this is a rare event compared with HDACi-induced G1 arrest. Inhibition of the cell cycle is a necessary event in cellular differentiation, and numerous papers reported on HDACi-related induction of differentiation in AML cell lines and AML cells derived from patients (Boissinot et al., 2012; Ryningen et al., 2007).
- **Tumour angiogenesis, metastasis and invasion.** HDACi have anti-angiogenic, anti-invasive and immunomodulatory activities *in vitro* and *in vivo* that can contribute to the inhibition of tumour development and progression. The anti-angiogenic properties of HDACi have been associated with decreased expression of pro-angiogenic genes including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), hypoxia-inducible factor-1α (HIF1α) (Sasakawa et al., 2003). In addition, HDACi have been shown to down-regulate expression of the chemokine (C-X-C motif) receptor 4 (CXCR4), which is important for the homing of bone-marrow progenitor and circulating endothelial cells to sites of angiogenesis. Finally, HDACi have been shown to suppress endothelial progenitor cell differentiation supporting a role for HDACi in suppressing neovascularization. The putative antimetastatic effect of

HDACi might be extended through suppression of matrix metalloproteinases (MMPs) (Liu et al., 2003).

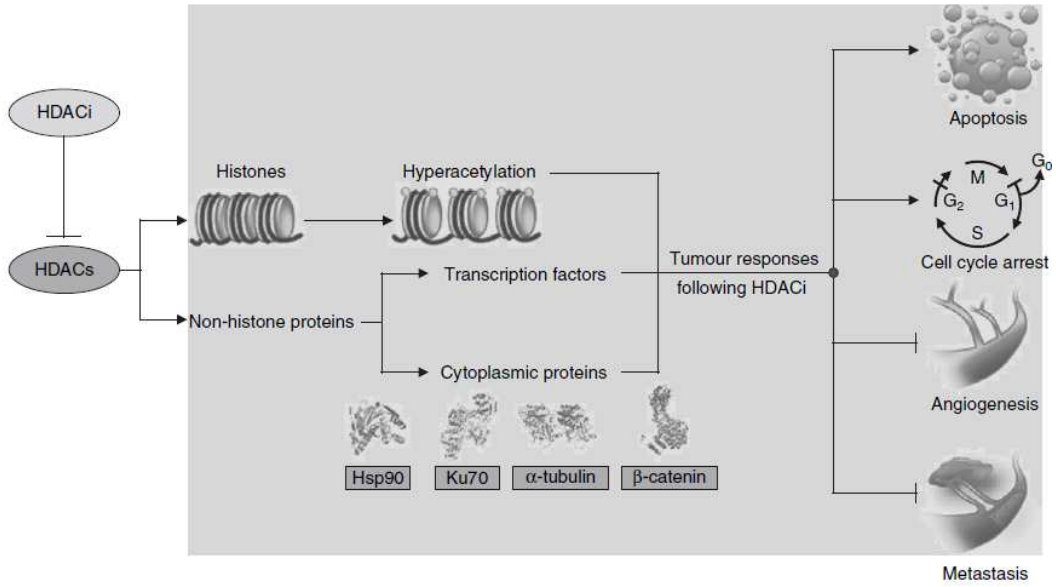


Fig.2: Schematic representation of anticancer effects of HDAC inhibitors (Ma et al., 2009).

Lee and colleagues investigated the selectivity of the HDACi Vorinostat in inducing cell death in cancer cells, but not in normal cells (Lee et al., 2010). Interestingly, Vorinostat was able to induce DNA double strand breaks (DSBs) both in normal than in tumour cells. Normal cells in contrast to cancer cells repair the DSBs despite continued culture with Vorinostat. In transformed cells, phosphorylated H2AX (γH2AX), a marker of DNA DSBs, increased with continued culture with Vorinostat, whereas in normal cells, this marker decreased with time. Further, they found that Vorinostat suppressed DNA DSB repair proteins (RAD50, MRE11) in cancer but not normal cells. The DNA damage is associated with cancer cell death and this can explain, in part, the selectivity of HDACi in causing cancer cell death.

1.3.3 Combination therapy involving HDACi

In addition to their intrinsic cytotoxic properties when tested as a single treatment, HDACi have been shown to induce additive cytotoxic effects when used in combination with conventional anticancer therapies, such as chemotherapy and

radiotherapy. Pre-clinical data in multiple cancer cell lines have shown the synergistic effects of HDACi in combination with topoisomerase I inhibitors (camptothecin, irinotecan, topotecan), topoisomerase II inhibitors (epirubicin, doxorubicin, etoposide, mitoxantrone) and other DNA-damaging agents (cisplatin, oxaliplatin, bleomycin) (Nolan et al., 2008). Concerning leukaemias, the HDACi Panobinostat potentiated the *in vivo* effects of vincristine and dexametasone in a mouse model of human ALL (Vilas-Zornoza et al., 2012). Regarding radiotherapy, there is evidence that HDACi decrease the cell's capacity to repair ionizing-radiations (IR)-induced DNA damage, both at the level of damage signalling and by affecting the major DNA repair pathways (Non Homologous End Joining and Homologous Recombination), in many different cell types *in vitro*. Tumour cells treated with various HDACi display prolonged resolution of IR-induced γH2AX foci, an indicator of impaired double strand breaks repair, which is due to impaired recruitment or lower quantities of repair proteins (Groselj et al., 2013). For example, in a subcutaneous mouse model of acute lymphoblastic leukaemia, treatment with Panobinostat resulted in an increase in γH2AX levels in tumour cells (Vilas-Zornoza et al., 2012).

1.3.4 HDACi in clinical trials

Currently, there are over 80 clinical trials investigating more than eleven different HDACi for both solid and haematological malignancies as monotherapies or in combination with various other antitumor agents. At present, two HDACi – Vorinostat (suberoylanilide hydroxamic acid, Zolinza®) and depsipeptide (romidepsin, Istodax®) – received approval from the US Food and Drug Administration (FDA) for treatment of refractory cutaneous T-cell lymphoma (CTCL), and more recently, depsipeptide has gained FDA approval for peripheral T-cell lymphoma (PTCL). Many efforts have been made to create chemically distinct HDACi, with several ongoing clinical trials in various malignancies, many of them focused on haematological pathologies, such as leukaemias, lymphomas, and myelodysplastic syndromes. In particular, Vorinostat was FDA-approved in 2006 for CTCL, which previously could not be treated by other drugs. FDA approval was based on two phase II clinical trials with a 30%

response rate in patients with CTCL. Vorinostat was generally well tolerated, with adverse side effects including diarrhea, fatigue, and nausea (Duvic et al., 2007). Similar responses have been observed in patients with relapsed non-Hodgkin's lymphoma and mantle-cell lymphoma (Kirschbaum et al., 2011). Numerous phase I and II clinical trials with HDACi in AML patients have been performed. In a phase I clinical trial, significant anti-leukaemia activity was observed in patients with AML, and Vorinostat effectively inhibited HDAC activity in peripheral blood and bone marrow blasts, even if no correlation between acetylated histone H3/H4 and clinical response was found (Garcia-Manero et al., 2008). Similar results in adults with refractory and relapsed acute leukaemias treated with HDACi MS-275 have been reported. In a phase I clinical trial, treatment with MS-275 induced increase in protein and histone H3/H4 acetylation, p21 expression, and caspase-3 activation in bone marrow mononuclear cells (Gojo et al., 2007). At variance with the promising clinical responses obtained in patients with haematological malignancies, response rates to HDACi in solid cancers have been ineffective or modest. Studies in relapsed or refractory breast, colorectal, or non-small-cell lung cancer showed no response. (Blumenschein et al., 2008; Vansteenkiste et al., 2008).

In addition to those mentioned earlier, some of the more recent HDACi that have been tested in patients include Abexinostat, Givinostat, and Mocetinostat.

In conclusion, although average results of HDACi in clinical trials have been modest, it is quite possible that subsets of patients could have a great benefit. Future studies are needed to identify possible predictive biomarkers of response to HDACi.

2. AIM OF THE STUDY

Several studies investigated both *in vitro* and *in vivo* effects of HDACi in T acute lymphoblastic leukaemia (T-ALL). Most of these studies, however, used established T-ALL cell lines and they did not identify whether specific signalling pathways are perturbed by HDACi. A better knowledge of the molecular events underlying HDACi effects will improve clinical application of these drugs. In particular, the characterization of specific HDACi targets among transcription factors commonly de-regulated in T-ALL, could help to identify subgroups of patients more responsive to this treatment. For this purpose, we analysed the effects of HDACi on transcription factors relevant in T-ALL pathogenesis using both established T-ALL cell lines and patient-derived T-ALL xenografts previously obtained in our laboratory. In particular, we focused on transcription factors that define four genetic subgroups of T-ALL, including TAL/LMO, TLX1 and TLX3. Furthermore, we included in our analysis members of the Notch family (NOTCH1 and NOTCH3) and c-MYB in view of their transversal role in T-ALL. Finally, we strengthened *in vitro* observations by using HDACi under investigation in clinical trials in preclinical models of T-ALL. The long-term goal of the project is to investigate the possibility that certain genetic subgroups of T-ALL could be better candidates for therapeutic treatment with HDACi.

3. MATERIALS AND METHODS

3.1 T-ALL xenografts establishment

Primary T-ALL cells (PD-TALL) were obtained from bone marrow of newly diagnosed pediatric patients, according to the guidelines of the local ethics committees. For xenografts establishment, 6- to 9-weeks-old mice were injected intravenously (i.v.) with 10 x 10^6 T-ALL cells in 300 µl of Dulbecco's Phosphate Buffer Saline (PBS). T-ALL engraftment was monitored by periodic blood drawings and flow cytometric analysis of CD5 and CD7 markers over a 5-month period. NOD/SCID mice were purchased from Charles River (Wilmington, MA). Procedures involving animals and their care conformed with institutional guidelines that comply with national and international laws and policies (EEC Council Directive 86/609, OJ L 358, 12 December, 1987). To test the effects of Givinostat (ITF2357) on leukemia cells, NOD/SCID mice were intraperitoneally (i.p.) injected with Givinostat (25mg/kg) or PEG400/H_2O (vehicle) 2 days after leukemic cells injection and administered five days a week. In all experiments, mice were inspected twice weekly to detect early signs and symptoms of leukemia and blood was drawn to measure T-ALL cell engraftment.

3.2 NOTCH1 and FBW7 mutational analysis

Genomic DNA was extracted from T-ALL cells derived from xenografts with Easy DNA kit (Life Technologies). NOTCH1 and FBW7 mutation analysis was performed as describe in (Sulis et al., 2008; Thompson et al., 2007) in collaboration with Prof. Adolfo Ferrando (Dept. of Pediatrics, Columbia University Medical Center, New York, USA).

3.3 Cell lines and *in vitro* culture conditions

The T-ALL cell lines MOLT-3 and JURKAT were purchased from ATCC. DND 41 cell lines were kindly provided by A. Ferrando (Columbia University). All T-ALL cell lines were cultured in RPMI 1640 (EuroClone, Milan, Italy) supplemented with 10% FCS (Fetal Bovine Serum, Life technologies, Paisley, UK), 10mM HEPES (Cambrex Bioscience, East Rutherford, NJ), 1% Sodium Pyruvate, 2 mM L-glutamine and 1% of antibiotic-antimycotic mix (Life Technologies). The human embryonic kidney epithelium cell line 293T was purchased from ATCC and cultured in Dulbecco modified Eagle medium (Euroclone), supplemented with 10% FCS, 10 mM HEPES and 1% of antibiotic-antimycotic mix (Life Technologies, Paisley, UK). Primary T-ALL cells derived from mice spleen were cultured in MEMα medium (Life Technologies) supplemented with IL7, SCF, FTL3 (Peprotech, Rocky Hill, NJ) and human insulin (Sigma Aldrich, Saint Luis, MO). T-ALL primary cells and cell lines were cultured *in vitro* with RPMI/MEMα alone (with the appropriate vehicle when necessary), or plus the following: 500 nM thricostatin A (Sigma Aldrich), 2 μm Givinostat (ITF 2357 - Italfarmaco, Milan, Italy), 500 mM cyclohexamide (Sigma Aldrich), 20 μM MG132 (Sigma Aldrich), 20 μM chloroquine (Sigma Aldrich). At the indicated time points, the cells were harvested and processed for assessment of cell viability, and RNA and protein extraction.

3.4 RNA extraction, reverse transcription PCR (RT-PCR) and quantitative RT-PCR (qRT-PCR)

Total RNA was isolated using TRIzol® Reagent according to manufacturer's instructions. cDNA was synthesized from 0.5 to 1 μg of total RNA using Super Script II Reverse Transcriptase Kit. Reverse transcription was followed by quantitative PCR using SYBR Green. mRNA PCRs were performed in an ABI Prism 7900HT Sequence Detection System. All reagents were obtained from Life Technologies. Results were analysed using the ΔΔCt method with normalization against β2-microglobulin expression. Primers used for q RT-PCR analysis was:

C-MYB-for: 5'-ACCTAGCCCAAGGGTGAACA-3';
C-MYB-rev: 5'-TCGAAGGATGACCAGTGGAA-3';

CR2-for: 5'-CTGCGGTTCAGTGTCCACAT-3';
CR2-rev: 5'-GGTGAAGCCAAACATGCAAGC-3' ;
DTX-1-for: 5'-GTGGGCTGATGCCTGTGAAT-3';
DTX-1-rev: 5'-CGAGCGTCCTCCTTCAGCAC-3';
HES1-for: 5'-GGCGGCTAAGGTGTTTGGAG-3';
HES-1 rev: 5'-GGAAGGTGACACTGCGTTGG-3';
NOTCH1-for: 5'-GTCAAGCCAAATATGTCAGC-3'
NOTCH1-rev: 5'-GCCGGATAAGGTGCCGGAG-3'
NOTCH3-for: 5'-CAAGGGTGAGAGCCTGATGG-3';
NOTCH3-rev: 5'- GAGTCCACTGACGGCAATCC-3';
pTα-for: 5'- ATGGTGGTGGTCTGCCTGGT-3';
pTα-rev: 5'-AGTTGGTCCAGGTGCCATCC-3';
β2-microglobulin-for 5'-TGCTGTCTCCATGTTTGATGTATCT-3';
β2-microglobulin-rev: 5'-TCTCTGCTCCCCACCTCTAAGT-3'.

Expression levels of target genes in short term treatment experiments were analysed by Real Time Ready custom panels (Roche Diagnostics, Penzberg, Germany).

3.5 Gene Expression profiling and classification of T-ALL xenografts

Total RNA was extracted from the spleen using Trizol® reagent (Life Technologies), according to the manufacturer's instruction. RNA concentration was determined using NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc, Wilmington, DE). The instruments provided the sample concentration in ng/μl and the absorbance of the sample at 260nm and 280nm. The ratio (260/280) ranging from 1.8 to 2.1 indicated good quality of RNA (ratio < 1.8 means protein contamination and ratio > 2.1 RNA degradation and truncated transcripts). RNA quality and purity control was assessed with the Agilent Bioanalyzed 2100 (Agilent Technologies, Waldbronn, Germany) using "Eukaryote total RNA Assay". To perform gene expression experiments, extremely high quality of total RNA was used.

Only RNA samples that passed the high quality controls were diluted to 100 ng in a total volume of 3 µl DEPC treated water to perform gene expression experiments. *In vitro* transcription, hybridization and biotin labelling were performed according to GeneChip 3'IVT Express kit protocol (Affymetrix, Santa Clara, CA). The Affymetrix GeneChip Scanner was used to measure all intensities of the signals of each probe set on the GeneChip and stores all signals in a .DAT file (Raw image). Integrated software converts all raw signals into numbers, which were stored in a .CEL file. All GEP profiles used in these experiments were assessed for their comparability and quality, using different quality controls: Scale Factor, number of present calls, internal probe calls, Poly-A controls and the ratio GAPDH/β-actin 3'/5'.

Microarray data (.CEL files) were analyzed using Command Expression Console (Affymetrix). R-Bioconductor (Version 2.15.3) was used to analyse the .CEL files data. Supervised classification (PAM_predictive analysis of microarrays) was used to construct a predictive algorithm able to classify samples for the main cytogenetic subgroups (*TALLMO, TLX1, TLX3, HOXA*) that characterize the T-ALL. Predictive algorithm was developed on a "training" data set, where the categories to which objects belonged were known and evaluated on an independent "test" data set, in which objects were assigned to previously defined categories.

3.6 Retroviral vectors production

Viral vectors were generated using 293T cell lines due to their high transfection capacity. In particular, cells were transiently transfected with 3 plasmids using calcium phosphate transfection method. The viral vectors produced in this way, belong to HIV-based vectors called SIN (self- inactivating). Plasmids used for viral production are listed below:

- A plasmid coding the transgene of interest;
- A packaging plasmid (gag-pol gpt), coding HIV *gag* and *pol* genes;
- pHCMV-G plasmid driving the expression of Vescicular Stomatitis Virus protein G (VSV-G). This protein of the envelope allows to extend the tropism of the virus.

Expression constructs used in this project:

- pCEG and pNOTCH3 CEG: NOTCH3 ICD retroviral over-expressing vectors;
- pMIGRI and pNOTCH1 MIGRI: NOTCH1 ICD retroviral over-expressing vectors.

3.7 Transduction of T-ALL cells

JURKAT cells were transduced with VSVG-pseudotyped NOTCH3-expressing or NOTCH1-expressing retroviruses. Briefly, $1x10^6$ cells were incubated at 37°C O.N. with $1x10^8$ virus particles and Protamine Sulfate. The day after, cells were washed and cultured in fresh medium. After expansion, cells were analyzed for high green fluorescent protein expression by flow cytometry analysis and used for caspase assay and western blot analysis.

3.8 Caspase assay

In rescue experiments, apoptosis was evaluated measuring caspase 3-7 activity with CaspaseGlo 3/7 assay kit (Promega, Madison, WI). These members of the cysteine aspartic acid-specific protease (caspase) family are key effectors of apoptosis in mammalian cells. The assay provides a luminogenic caspase-3/7 substrate, which contains the tetrapeptide sequence DEVD, in a reagent optimized for caspase activity. Adding a single Caspase-Glo 3/7 reagent results in cell lysis, followed by caspase cleavage of the substrate and generation of a luminescent signal, produced by luciferase (Figure 3). Luminescence is proportional to the amount of caspase activity present.

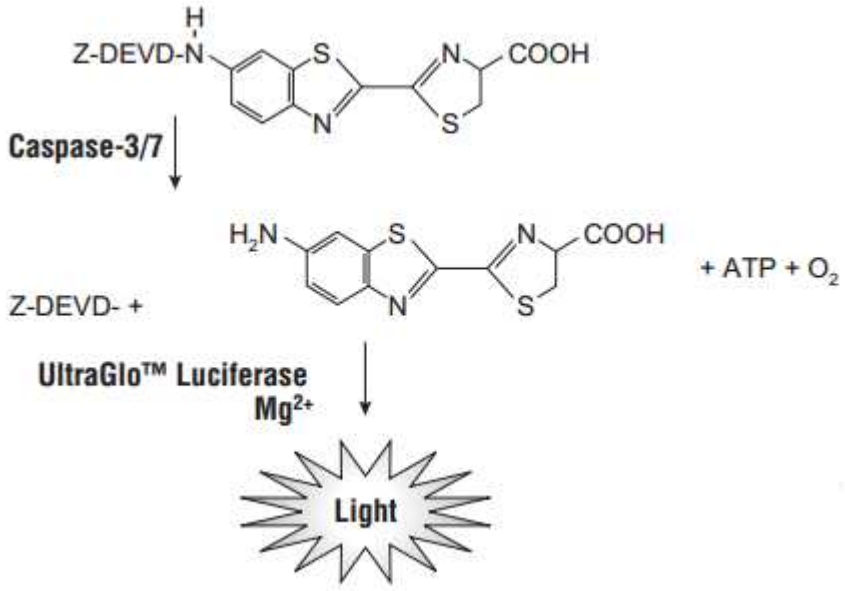


Fig. 3: Caspase-3/7 cleavage of the luminogenic substrate containing the DEVD sequence. Following caspase cleavage, a substrate for luciferase (aminoluciferin) is released, resulting in the luciferase reaction and in the production of light.

3.9 MTS assay

Proliferation rate of T-ALL cells after HDAC inhibition were measured using CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega). The MTS tetrazolium compound (Owen's reagent) is bioreduced by cells into a colored formazan product that is soluble in tissue culture medium (Figure 4). This conversion is presumably accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells. The quantity of formazan product as measured by the absorbance at 490nm is directly proportional to the number of living cells in culture.

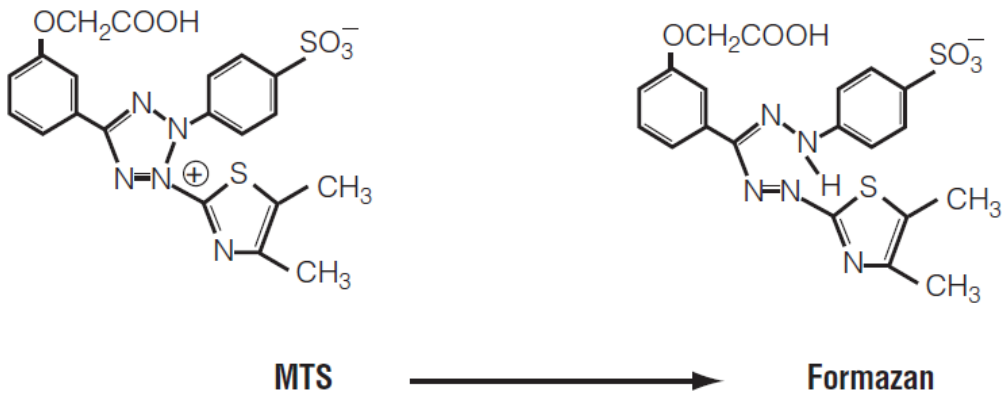


Figure 4: Structures of MTS tetrazolium and its formazan product.

3.10 Cytofluorimetric analysis

Anti-human FITC-conjugated CD5 and PE-Cy5-conjugated CD7 antibodies (Coulter, Fullerton, CA) were used for the detection of T-ALL cells in blood, spleen and bone marrow samples. Apoptosis was evaluated by the Annexin-V-FLUOS Staining Kit (Roche Diagnostics). To test the effective transduction of T-ALL cells with retroviral vectors, percentage of green fluorescent protein (GFP) positive cells was measured 48 h after transduction by cytometric analysis. Indeed, both expression plasmids (N3 CEG, N1 MIGRI and control) have GFP reporter gene. Samples were analyzed on Beckman Coulter EPICS-XL Flow Cytometer (Coulter) or BD LSRII Flow Cytometer (BD Biosciences, San Jose, CA).

3.11 Western Blot analysis

Cells were resuspended in lysis buffer (NP-40 1%, NaCl 150 mM, Tris HCl pH7.5 50 mM, EDTA 2mM, NaF, Na_3VO_4 and protease inhibitor cocktail) and lysates obtained were quantified using Quantum protein Assay (Euroclone). About 30 µg of proteins were denaturated and loaded in a midi polyacrylamide gel 4-12% (Life Tecnologies). Separated proteins were transferred for 2 h at 400mA on a nitrocellulose membrane (GE Health Care, Glattbrugg, Switzerland). Membranes were saturated O.N. at 4°C with PBS - 0,1% Tween - 5% - milk and then incubated with primary antibody according to manufacturer's instructions.

In this work, these primary antibodies were used:

- Rabbit anti ACTIN (Sigma Aldrich);
- Mouse anti TUBULIN (Sigma Aldrich);
- Rabbit anti PARP (Cell Signalling, Danvers, MA);
- Rabbit anti NOTCH3 (Abcam, Cambridge, UK);
- Rabbit anti NOTCH1 C44H11(Cell Signalling);
- Mouse anti c-MYB (Merck-Millipore, Billerica, MA);
- Mouse anti c-MYC (Merck-Millipore);
- Mouse anti Acetylated α TUBULIN (Santa Cruz Biotechnologies, Dallas, Texas);
- Mouse anti TAL1 (Merck-Millipore);
- Rabbit anti LMO2 (Abcam);
- Rabbit anti TLX1 (Sigma Aldrich);
- Mouse anti TLX3 (Sigma Aldrich);
- Rabbit anti NOTCH1 ICD Val1744 (Cell Signalling);
- Rabbit anti p16 (Abcam).

Secondary antibodies (GE Healthcare) are conjugated with horseradish peroxidase. When reaction mix, containing Luminol, joins with peroxidase, the substrate is oxidated resulting in emission of light at 425nm. For detection, Western Lightning plus ECL reagents (Perkin Elmer, Waltham, MA) were used. The signal emitted by the reaction was acquired by acquisition imagine system ChemiDoc XRS (Bio Rad, Hercules, CA).

3.12 Statistical analysis

Results were expressed as mean value ± SD. Statistical analysis of data was performed using Student's t-test or Mann-Whitney test. Differences were considered statistically significant when $P \leq 0.05$.

4. RESULTS

4.1 Functional effects of HDAC inhibition in T-ALL cells

HDACi have moderate to strong therapeutic activity in several haematological malignancies and according to previous studies, HDACi are potent inducers of apoptosis in leukaemia cells (Aldana-Masangkay et al., 2011; Vilas-Zornoza et al., 2012). In order to investigate the therapeutic potential of HDACi in the context of T-ALL, we treated with the pan-HDAC inhibitor Tricostatin A (TSA) 0.5 μM for 24 h a panel of established T-ALL cell lines (DND 41, MOLT3 and JURKAT) and xenografts cells derived from primary leukemic samples from patients. The concentration of TSA was based on published data on leukaemia cells *in vitro* (Chambers et al., 2003; Palermo et al., 2012). We successfully established mice xenografts and treated them *ex vivo* with HDACi. Briefly, immunodeficient NOD/SCID mice were injected intravenously with $1x10^7$ cells/mouse derived from bone marrow of paediatric T-ALL patients. In order to track tumour burden, regular blood drawings and cytofluorimetric analysis were performed to analyse the percentage of leukemic cells in circulation. At the appearance of signs of illness, mice were sacrificed. The leukemic cells were collected from the spleen, infiltrated > 90%, and used for *in vitro* studies with HDAC inhibitors. We chose to analyse the effects of HDACi on seven xenografts with distinct molecular and clinical phenotypes such as NOTCH1 mutation status, genetic subgroup, prednisone sensitivity and MRD risk (See table 7).

SampleID	Age (years)	Phenotype	MRD risk	PGR/PPR	NOTCH1 status	FBW7 staus	Genetic subgroup
PD-TALL6	13	T Int	MR	PGR	wt	wt	TAL/LMO
PD-TALL8	3	T Int	MR	PPR	HD+PEST mut.	wt	TLX1
PD-TALL9	9	Early T	HR	Deceased	wt	wt	TAL/LMO
PD-TALL10	10	Early T	MR	PGR	HD mut.	mut.	ND
PD-TALL12	4	Early T	MR	PGR	PEST mut.	wt	TAL/LMO
PD-TALL16	5	T Mat	MR	PPR	wt	wt	TLX3
PD-TALL19	16	Early T	Relapse	Relapse	HD + TAD mut.	mut.	ND

Table 7: Paediatric T-ALL patients were classified for differentiation phenotype, risk (according to the MRD classification) and response to therapy. All patients were aged between 3-16 years. Mutational status of NOTCH1 and FBW7 and the genetic subgroup are also reported. [MR= medium risk, HR= high risk; PGR= prednisone good responder, PPR= prednisone poor responder; HD= heterodimerization domain, PEST= proline-glutamic acid-serine-threonine-rich domain, TAD= C-terminal transcription activation domain]

Inhibition of HDACs exerted strong pro-apoptotic effects in all the cell lines and T-ALL cells from xenografts tested (Fig.5). These effects were not associated with the mutational status of NOTCH of T-ALL cells (Fig. 5B).

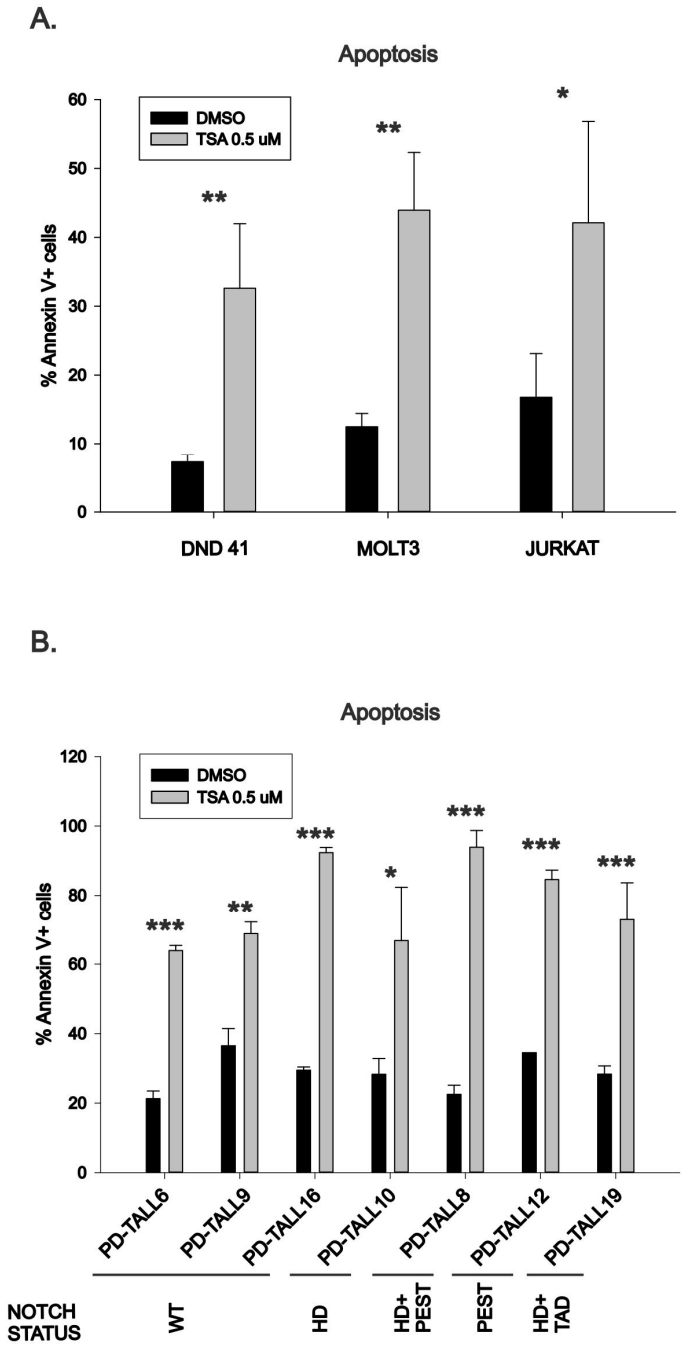


Fig.5: HDACi-mediated induction of apoptosis in T-ALL cells. T-ALL cell lines **(A)** or primary T-ALL cells **(B)** were treated *in vitro* with TSA (0.5 uM) for 24 h. Induction of apoptosis was measured by flow cytometric analysis of Annexin V staining (* P<0.05, ** P<0.01, *** P <0.001, mean ± SD of three independent experiments).

To study the effect of HDAC blockade on proliferation in our cell contest, we assessed MOLT3 proliferation rate using both a metabolic assay (MTS) and cell count. As shown in Fig.6, MOLT3 treated with TSA decreased proliferation compared to controls both at 24 h and 30 h. Later time points were not considered due to the reduced viability of the cells.

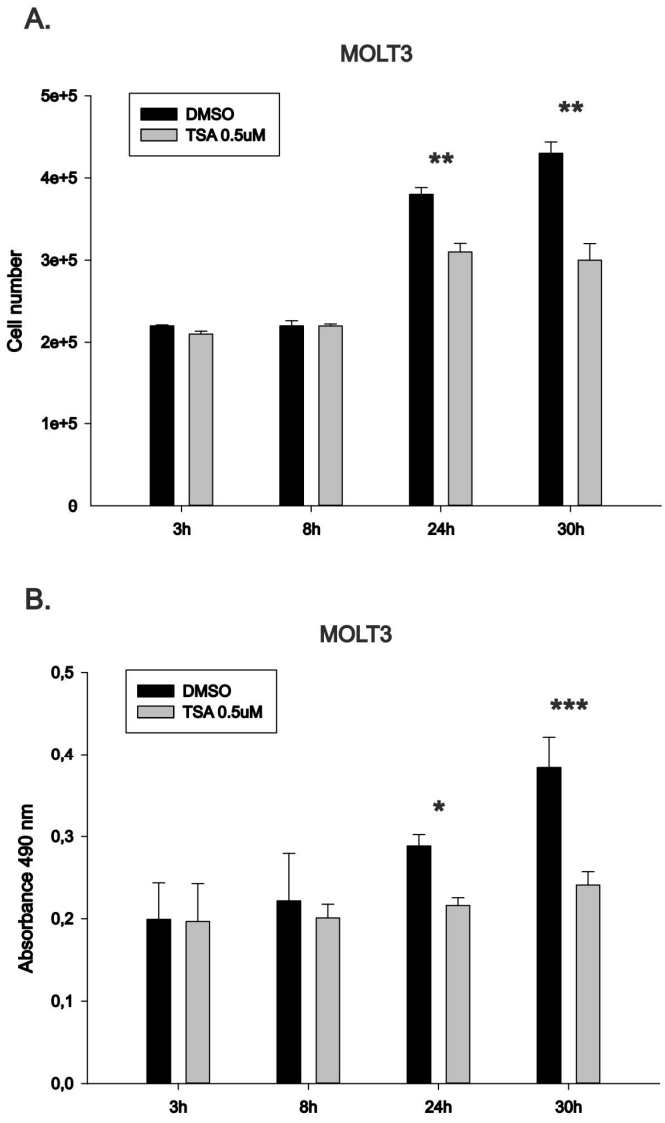


Fig 6: HDACi-induced inhibition of proliferation in MOLT3. MOLT3 were treated with TSA (0.5 µM). Cell number **(A)** and metabolic activity **(B)** were assessed at 3, 8, 24 and 30 h respectively by cell count and MTS assay (* P<0.05, ** P<0.01, *** P <0.001, mean ± SD of three independent experiments).

4.2 HDAC inhibitor TSA down-regulates relevant T-ALL transcription factors protein levels

In order to investigate the effect of HDACi on the expression of transcription factors associated to T-ALL leukaemogenesis, the T-ALL cell lines DND 41, MOLT3 and JURKAT were treated *in vitro* with TSA 0.5 μM. After 16 h of treatment, whole cell lysates were extracted and analysed for the protein levels of a selected panel of transcription factors (NOTCH1, NOTCH3, c-MYB, TLX1, TLX3, LMO1, LMO2 and TAL1) by western blot. Accumulation of acetylated α tubulin, a direct target of HDAC6, was used as a read-out of HDAC activity inhibition. As expected, treatment with TSA for 16 h resulted in the increase of acetylated α tubulin (Fig.7).

Interestingly, we observed that TSA decreased protein levels of NOTCH1, NOTCH3, c-MYB and TAL1 in all the cell lines tested. On the contrary, LMO2, TLX3 and TLX1 levels were not significantly affected by HDAC inhibition (Fig. 7).

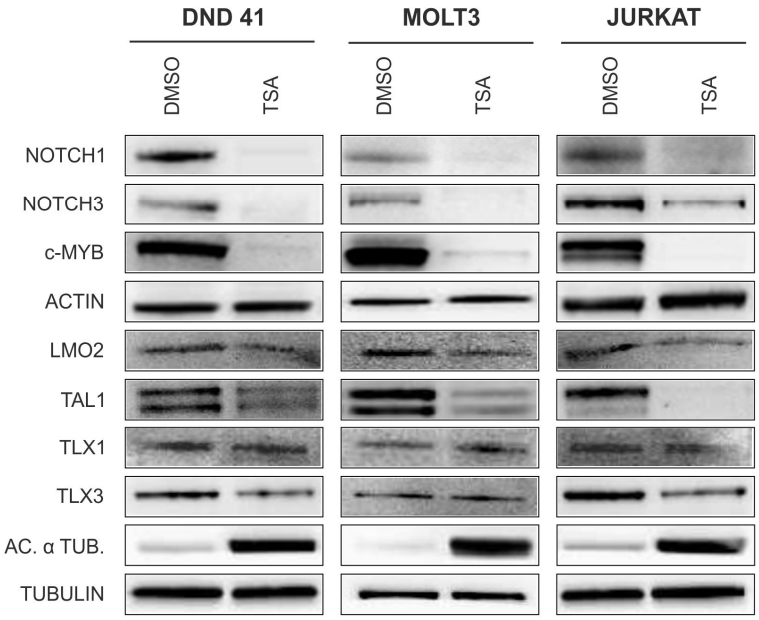


Fig.7: Effects of HDACi on transcription factors relevant in T-ALL. DND 41, MOLT3 and JURKAT were treated with TSA (0.5 μM) for 16 h and protein levels were analysed by western blot. The housekeeping protein ACTIN was used as loading control. Tubulin acetylation was used as a marker of HDAC inhibition.

To confirm the results obtained, we treated *ex vivo* T-ALL xenografts cells with TSA. After 16 h of treatment, NOTCH1, NOTCH3, c-MYB and TAL1 protein level displayed a marked reduction, whereas TLX3, TLX1 and LMO2 expression was not altered by TSA (Fig. 8)

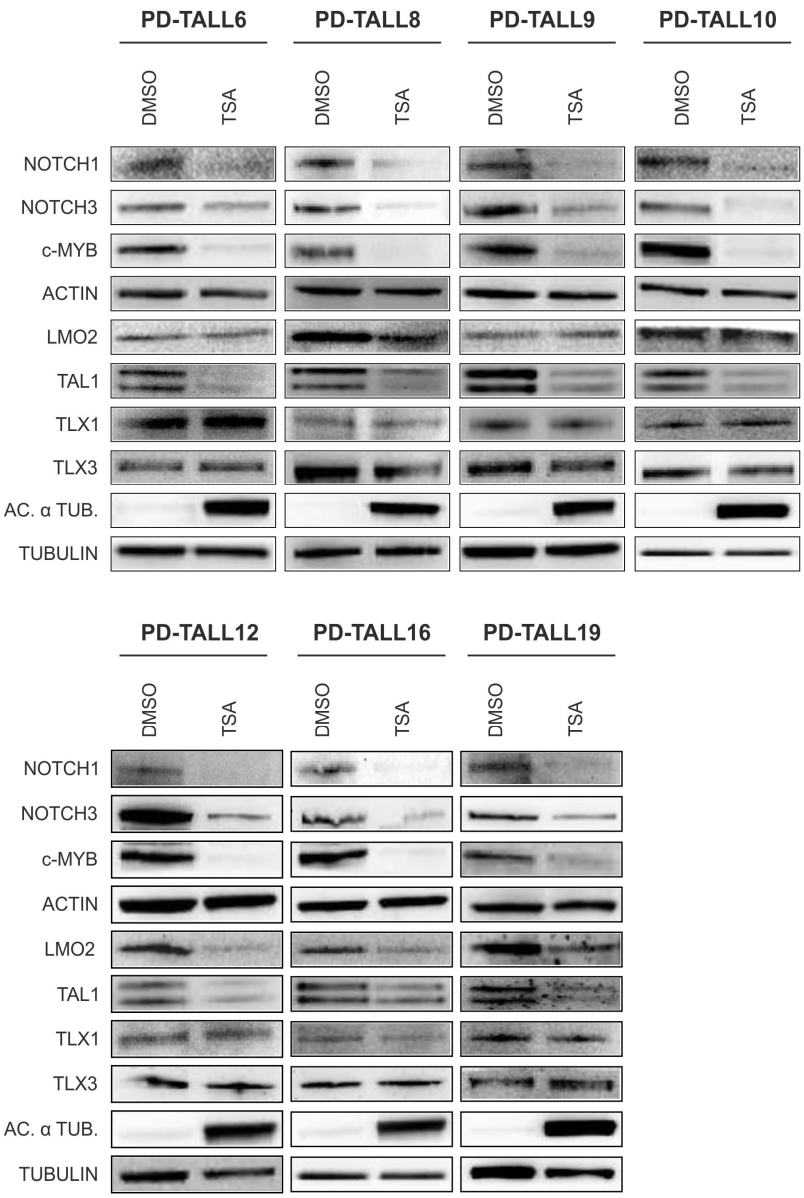


Fig.8: Effects of TSA treatment on transcription factors expressed in primary T-ALL cells. T-ALL cells were recovered from spleen of xenografted mice and were treated *in vitro* with TSA (0.5 µM) for 16 h. Protein levels were analysed by western blot.

4.3 Differential effects of HDAC inhibition on mRNA expression of NOTCH1, NOTCH3 and c-MYB

To investigate whether the TSA-induced suppressive effect at protein level was associated with inhibition of transcription, we analysed the mRNA levels of *NOTCH1*, *NOTCH3* and *c-MYB* upon HDACi treatment. TAL1 was excluded from this analysis, as its transcription has been previously reported to be suppressed by HDACi in ALL cells (Cardoso et al., 2011). Interestingly, *c-MYB* mRNA displayed more than 80% reduction in all cell lines tested. On the contrary, we obtained heterogeneous results for *NOTCH1* and *NOTCH3*. In fact, upon TSA-treatment mRNA expression of the two NOTCH receptors was significantly altered in DND 41 but not in MOLT3 and in JURKAT cells (Fig. 9A). Similar results were obtained with three representative human T-ALL xenografts (PD-TALL6, PD-TALL8 and PD-TALL9) (Fig. 9B). This result suggests that NOTCH1 and NOTCH3 could be either post-transcriptionally and/or post-translationally regulated by TSA depending on the cell line analysed.

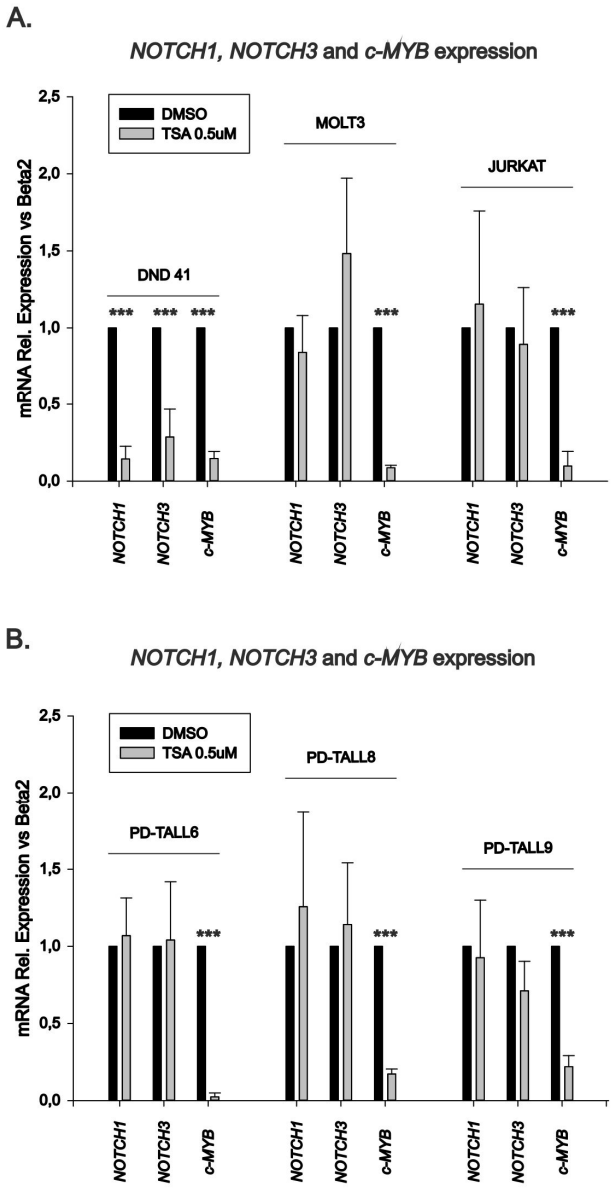


Fig 9: Transcript levels of *NOTCH1*, *NOTCH3* and *c-MYB* in T-ALL cell lines after HDAC inhibition. T-ALL cell lines **(A)** or primary T-ALL cells **(B)** were treated with TSA (0.5 uM) for 16 h and mRNA levels of *NOTCH1*, *NOTCH3* and *c-MYB* were analysed by qRT-PCR. Statistically significant differences are indicated (* P<0.05, ** P<0.01, *** P <0.001, mean ± SD of three independent experiments).

In line with western blot analysis, blockade of HDAC activity decreased the expression of NOTCH target transcripts, such as *pTα, CR2* and *DTX-1*, thus indicating attenuation of NOTCH signalling (Fig 10).

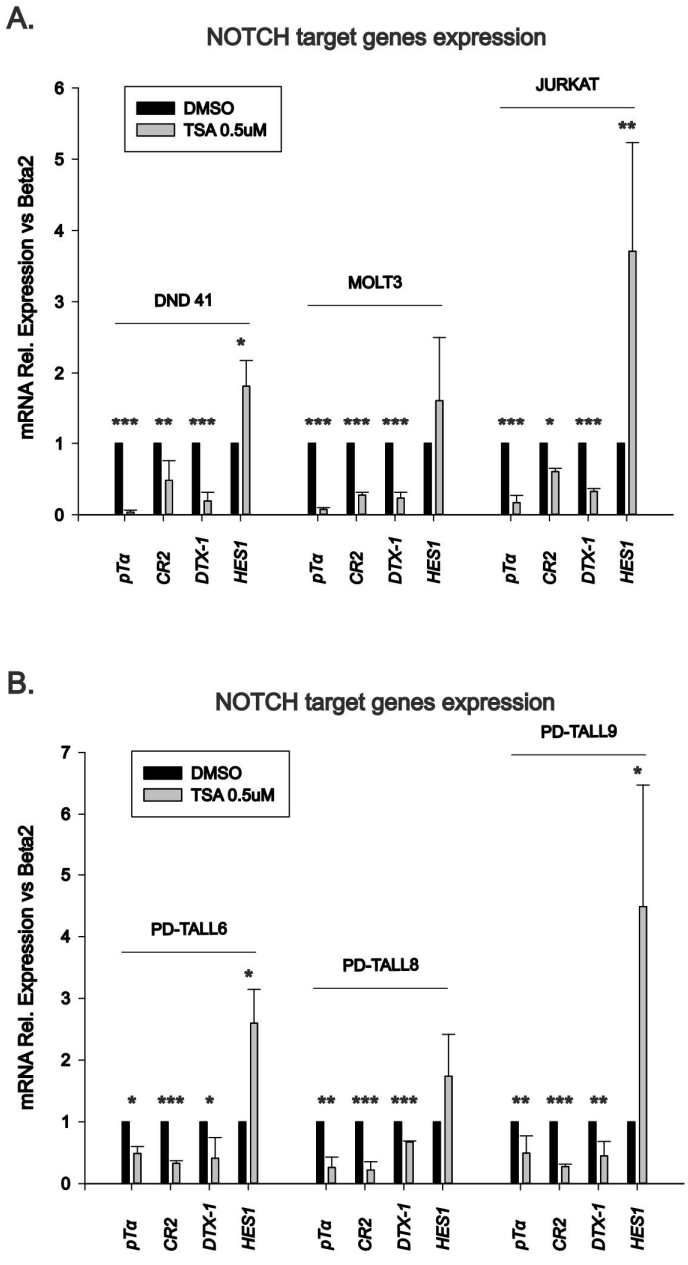


Fig 10: NOTCH target genes expression after TSA treatment. T-**ALL c**ell lines **(A)** or T-ALL cells from xenografts **(B)** were treated with TSA (0.5 μM) for 16 h and mRNA levels of NOTCH target genes (*pTα, CR2, DTX-1, HES1*) were analysed by qRT-PCR (* P<0.05, ** P<0.01, *** P <0.001, mean ± SD of three independent experiments).

4.4 Forced NOTCH1 and NOTCH3 expression partially prevents T-ALL cell death induced by HDAC inhibition

The transcription factors NOTCH1, NOTCH3 and c-MYB are known to play a fundamental role in cell death evasion (Lahortiga et al., 2007; Lewis et al., 2007; Sarvaiya et al., 2012; Zhou et al., 2011). Based on this, we next investigated whether down-regulation of these transcription factors was the basis of TSA-induced apoptosis in T-ALL cells. To this aim, we analysed pro-apoptotic effects of TSA upon forced expression of NOTCH1 or NOTCH3 in T-ALL cells. We transduced JURKAT cells with retroviral vectors driving the expression of NOTCH1 and NOTCH3 intracellular domain (ICD), the constitutively active form of the transcription factor (Fig.11A). Forced expression of NOTCH1 and NOTCH3 significantly reversed the induction of apoptosis mediated by TSA in T-ALL cells, measured by caspase assay and the ratio cleaved:unclived PARP (Fig. 11 B and C). These results suggest that HDACi promote apoptosis in T-ALL cells partly via suppression of NOTCH1 and NOTCH3 activity. Similar rescue experiments are undergoing with c-MYB.

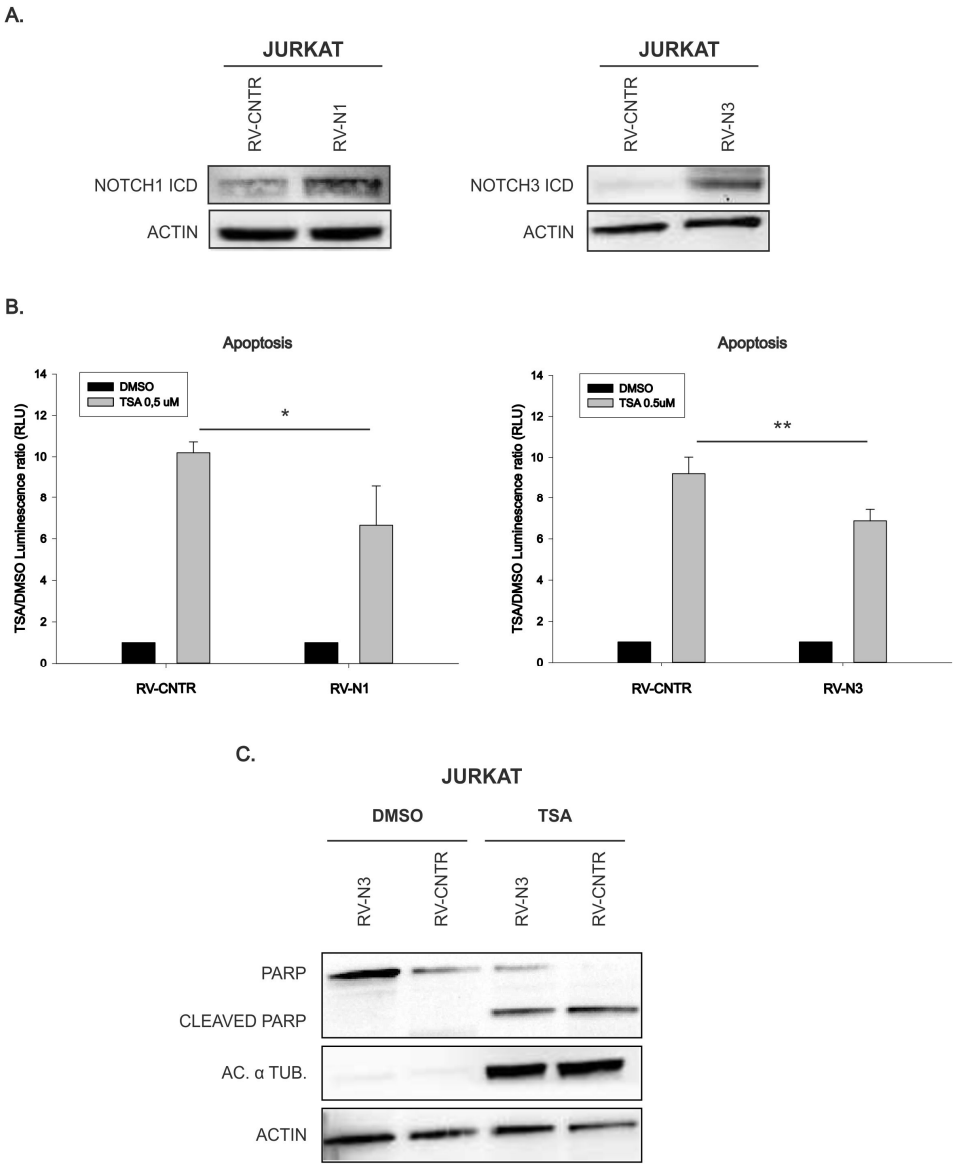


Fig. 11: Forced expression of NOTCH1 and NOTCH3 ICD reduced apoptosis in JURKAT cells. A) JURKAT were transduced with a retroviral vector expressing NOTCH1 or NOTCH3 intracellular domain (RV-N1 and RV-N3) or with the control vector (RV-CNTR). Over-expression was monitored by western blot. **B)** JURKAT cells transduced by RV-CNTR or RV-N1/RV-N3 vectors were treated with TSA 0.5 μM for 24 h. Apoptosis was monitored by caspase 3-7 assay and, for NOTCH3, by western blot analysis of cleaved PARP **(C)**. TSA/DMSO luminescence ratio are reported (**p<0.01, three independent experiments).

4.5 HDACi-mediated NOTCH3 down-regulation in T-ALL cells is dependent on increased protein degradation

Several reports indicate that HDACi can induce degradation of oncogenes and other cellular proteins by affecting protein stability. To test whether protein degradation has a role in the effects of HDACi on c-MYB and NOTCH3 protein levels, we treated MOLT3 cells with cyclo-heximide (CHX), a well-known protein translation inhibitor. The half-life of c-MYB, which is roughly 8 h in MOLT3 cells, was not significantly altered by treatment with TSA, indicating that this HDACi lacks major effects on c-MYB protein stability. In contrast, NOTCH3 protein levels decreased faster upon CHX treatment in the presence of TSA (Fig. 12). This result indicates that HDACi affects NOTCH3 protein stability, implying a post-translational mechanism of regulation.

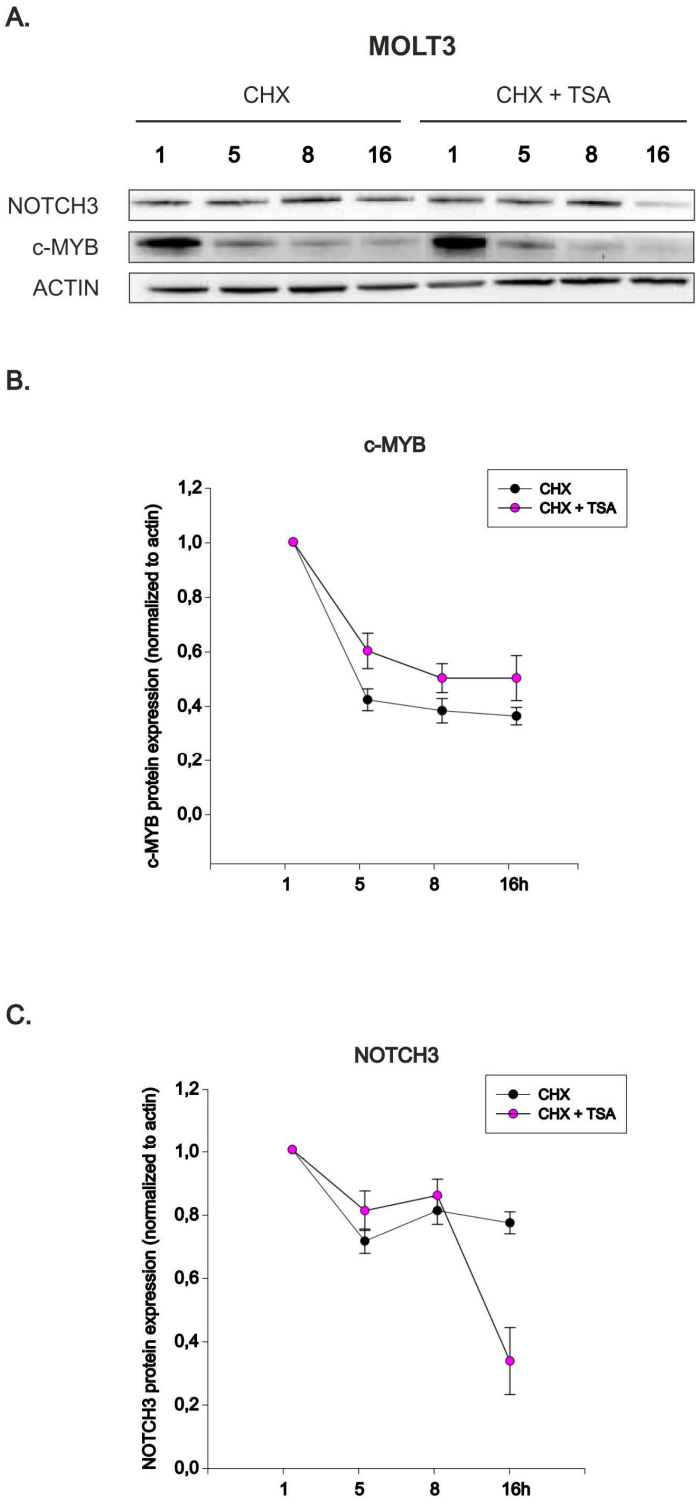


Fig.12: HDACi increased NOTCH3 protein degradation. A) MOLT3 were treated with CHX (500 µM) or with CHX (500 µM) and TSA (0.5 µM). At 1, 5, 8 and 16 h protein levels of c-Myb and NOTCH3 were analysed by Western Blot. A representative Blot is reported. C-MYB **(B)** and NOTCH3 **(C)** protein expression were measured by densitometric analysis and normalized to ACTIN. The graphs represent the mean of three independent experiments (**p<0.01).

4.6 HDACi-mediated NOTCH1 and NOTCH3 protein down-regulation in T-ALL cells is driven by lysosomal degradation

In mammalian cells, two major protein degradation pathways are known: the proteasome, which degrades mainly cytosolic proteins, and the lysosome, which accounts for membrane proteins turnover (Clague and Urbe, 2010). To investigate the molecular mechanism underlying increased NOTCH protein degradation, we treated MOLT3 cells with proteasome or lysosome inhibitors. Protein levels of the full-length NOTCH receptors were rescued using the lysosome inhibitor chloroquine (CHL), suggesting involvement of the endocytic pathway, whereas the proteasome inhibitor MG132 had minimal effects on NOTCH1 full-length levels and further reduced NOTCH3 full-length levels. As expected based on our previous findings, lysosome inhibition did not affect c-MYB protein levels (Fig.13 A). To test inhibition of proteasomal activity by MG132, we treated MOLT3 with MG132 and analysed protein levels of the transcription factor C-MYC, which is normally degraded by proteasome. As expected, C-MYC protein levels increased after proteasomal inhibition (Fig. 13 B).

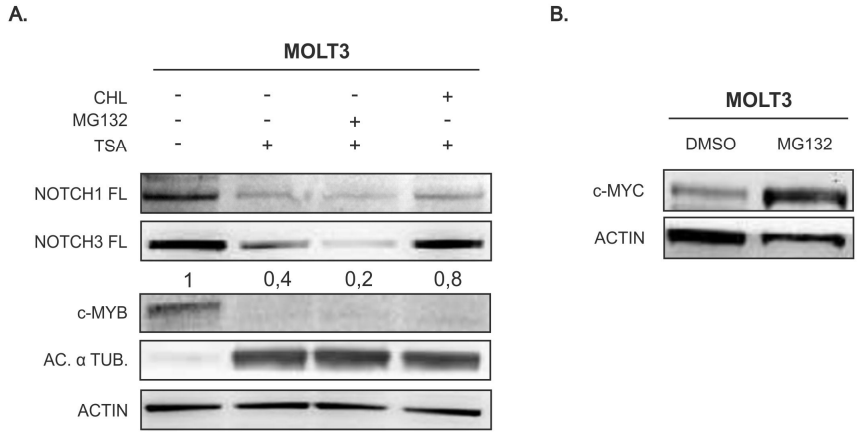


Fig.13: Lysosome inhibitor impaired NOTCH1 and NOTCH3 protein degradation. A) MOLT3 were treated with TSA 0.5 μM, CHL 20 μM or MG132 20 μM for 16 h and western blot analysis were performed. Numbers below the bands indicate densitometric analysis of NOTCH3 normalized to ACTIN. **B)** As control of MG132 activity, MOLT3 were treated with MG132 (20 μM for 8 h) and c-MYC protein levels were analysed by western blot. FL, full length.

4.7 Assessment of Givinostat effects on T-ALL cells in vitro

We sought to confirm the anti-tumour activity of TSA, using Givinostat, a pan-HDACi under investigation in clinical trials. Givinostat has been tested in phase II clinical trials for several haematological malignancies (including relapsed leukaemias and myelomas), and has been granted orphan drug designation in the EU for treatment of systemic juvenile idiopathic arthritis and polycythaemia vera. We first assessed the *in vitro* activity of Givinostat on T-ALL cell lines and primary T-ALL cells from xenografts. We treated cells with Givinostat 2 μm, according to Italfarmaco's instructions. Similarly to TSA, treatment with Givinostat determined a substantial reduction of NOTCH1, NOTCH3, c-MYB and TAL1 protein levels. In contrast, LMO2, TLX1 and TLX3 levels were apparently not modulated (Fig.14). In addition, in some cases we analysed protein levels of p16INK, known as CDKN2A (Cyclin-dependent kinase inhibitor 2A), a tumour suppressor protein, implicated in the regulation of cell cycle. Both in T-ALL cell lines and primary cells (Fig.12), p16 INK protein levels increased upon Givinostat treatment.

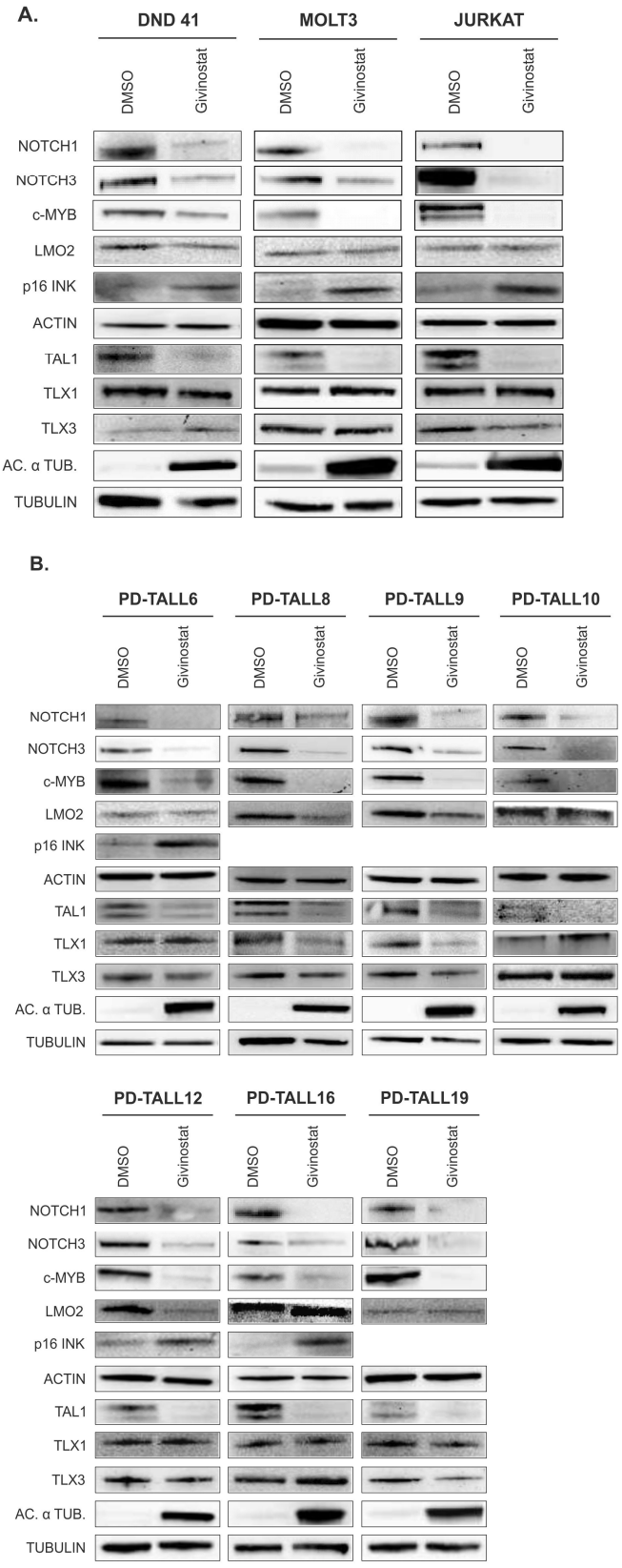


Fig.14: Effect of Givinostat on transcription factors expressed in T-ALL cells. T-ALL cell lines **(A)** and primary T-ALL cells from xenografts **(B)** were treated in vitro with Givinostat (2 μM) for 16 h and protein levels were analysed by western blot.

Finally, T-ALL cell lines and primary cells treated with Givinostat invariably showed increased apoptosis and impaired cell cycle progression (Fig.15).

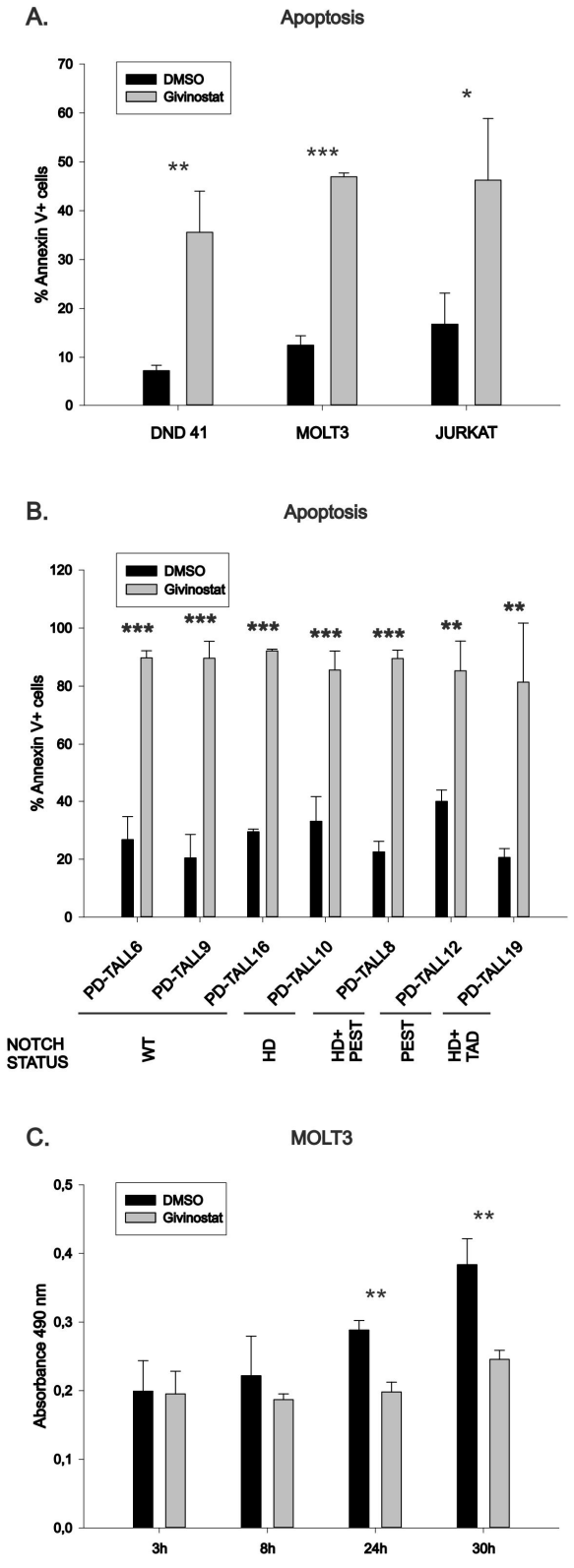


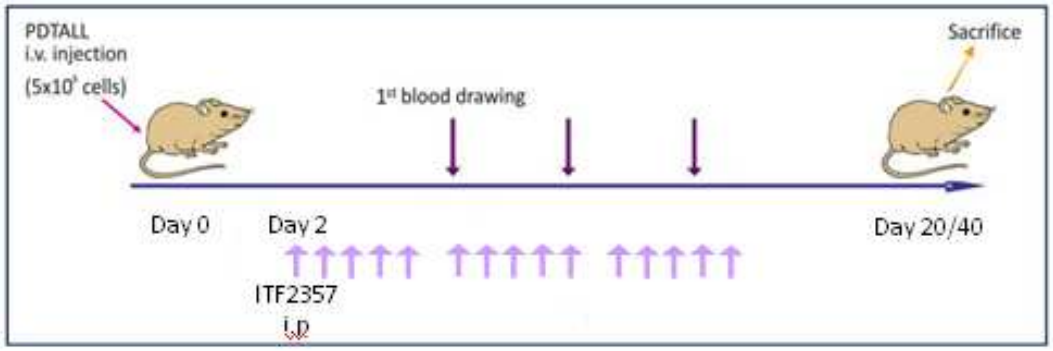
Fig.15: Givinostat increased apoptosis and impaired proliferation of T-ALL cells. T-ALL cell lines **(A)** or primary T-ALL cells **(B)** were treated *in vitro* with Givinostat (2 μM) for 24 h. Levels of apoptotic cells were measured by flow cytometric analysis of annexin V staining. (* P<0.05, ** P<0.01, *** P <0.001, three independent experiments). **C)** MOLT3 were treated with Givinostat (2 μM). At 3, 8, 24 and 30 h metabolic activity (an indirect reed-out of cells number) was analysed by MTS assay (** P<0.01, three independent experiments).

4.8 Therapeutic effects of Givinostat in T-ALL xenografts

We next asked whether primary samples belonging to different T-ALL subgroups could respond differentially to long term treatment with Givinostat *in vivo*, considering its effect on the expression of relevant transcription factors. In particular, for these experiments, we choose the following four xenografts belonging to different T-ALL genetic subgroups: PD-TALL12 (TAL-LMO), PD-TALL9 (TAL-LMO), PD-TALL8 (TLX1) and PD-TALL16 (TLX3). Since TAL-LMO subgroup is the most represented subgroup in T-ALL, it was easier to obtain xenografts belonging to this subgroup. On the other hand, since none of the xenografts established in our laboratory belongs to the HOXA subgroup, it was not possible to study the influence of HDACi treatment on this specific genetic subset.

T-ALL cells were injected i.v. in NOD/SCID mice at $5x10^6$ cells/mouse. Givinostat (25mg/kg) or PEG400/H_20 (vehicle) were administrated 5 days per week and treatment started 2 days after cell injection. The levels of blasts in the blood, spleen and bone marrow were evaluated by flow cytometric analysis (staining with anti-CD5 and anti-CD7, characteristic markers used to identify human T-ALL cells). At the appearance of signs of illness in control mice, both groups were sacrificed and therapeutic response was evaluated by analysis of percentage of leukemic cells and levels of apoptosis in bone marrow and spleen.

The experimental design is reported below.



The therapeutic effects of Givinostat were very variable among the four xenografts tested. PD-TALL8 and PD-TALL16 were best responders to the

treatment while PD-TALL12 and PD-TALL9 showed respectively partial and no measurable response. In particular, PD-TALL8 (TLX1) treated mice displayed a significant reduction in the percentage of $CD7^+$ cells in peripheral blood (Fig. 16 A) as well as in spleen and bone marrow at sacrifice (Fig. 16 B) compared to controls. Treated mice showed an increased in apoptosis in the spleen and albeit less prominent, in the bone marrow, consisting with the pro-apoptotic effects of Givinostat *in vitro* (Fig. 16 C). At sacrifice, spleen size was comparable in treated and control mice (Fig. 16 D).

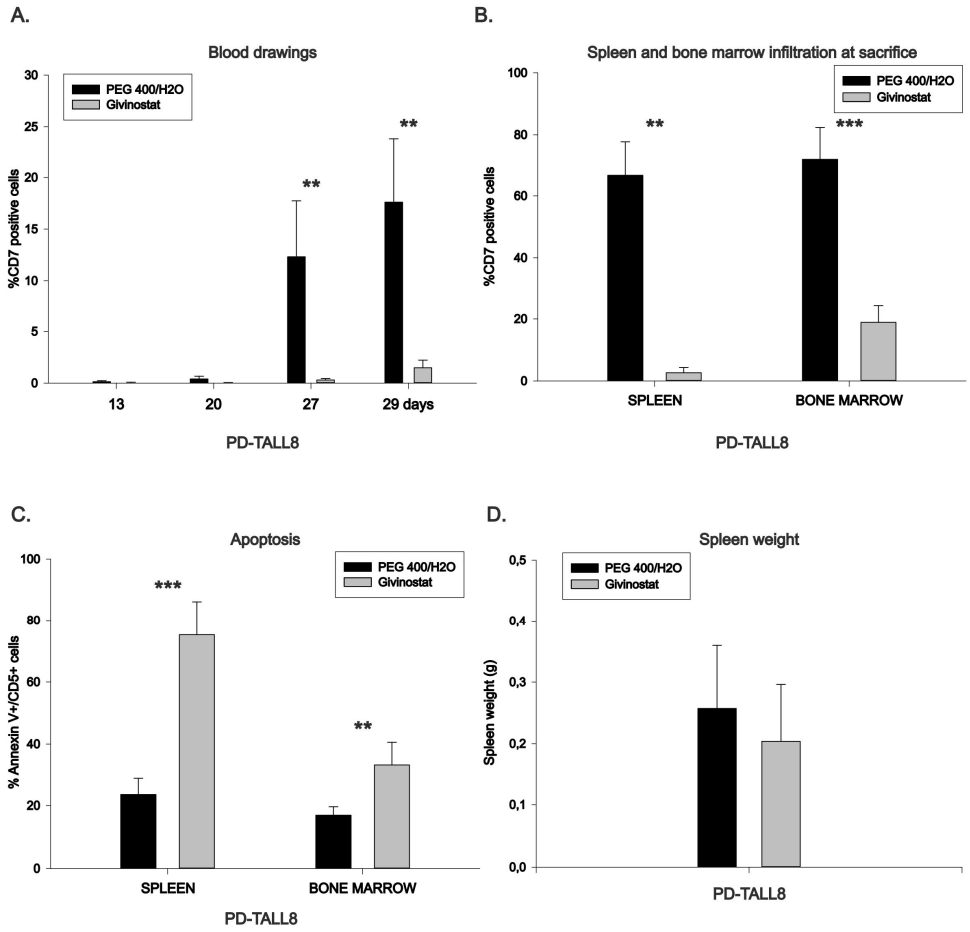


Fig.16: Therapeutic effects of Givinostat in PD-TALL8 xenografts (TLX1). A) Measurement of circulating blasts by flow cytometry in the blood. **B)** Percentage of leukemic cells in the spleen and bone marrow at sacrifice. **C)** At sacrifice, levels of apoptotic leukemic cells in the spleen and in the bone marrow were measured by annexin V labelling and flow cytometric analysis (n=6 mice/group) (* P<0.05, ** P<0.01, *** P <0.001).

PD-TALL16 (TLX3) also responded well to treatment. Also in this case, treated mice displayed a significant reduction of circulating blasts and in the percentage of infiltrating cells in spleen and bone marrow (Fig.17 A and B). Apoptosis levels in spleen and bone marrow increased in treated mice and were associated with a

significant decrease in spleen weight (Fig.17 C and D). As for PD-TALL8, the effect of treatment was more pronounced in the spleen than in the bone marrow.

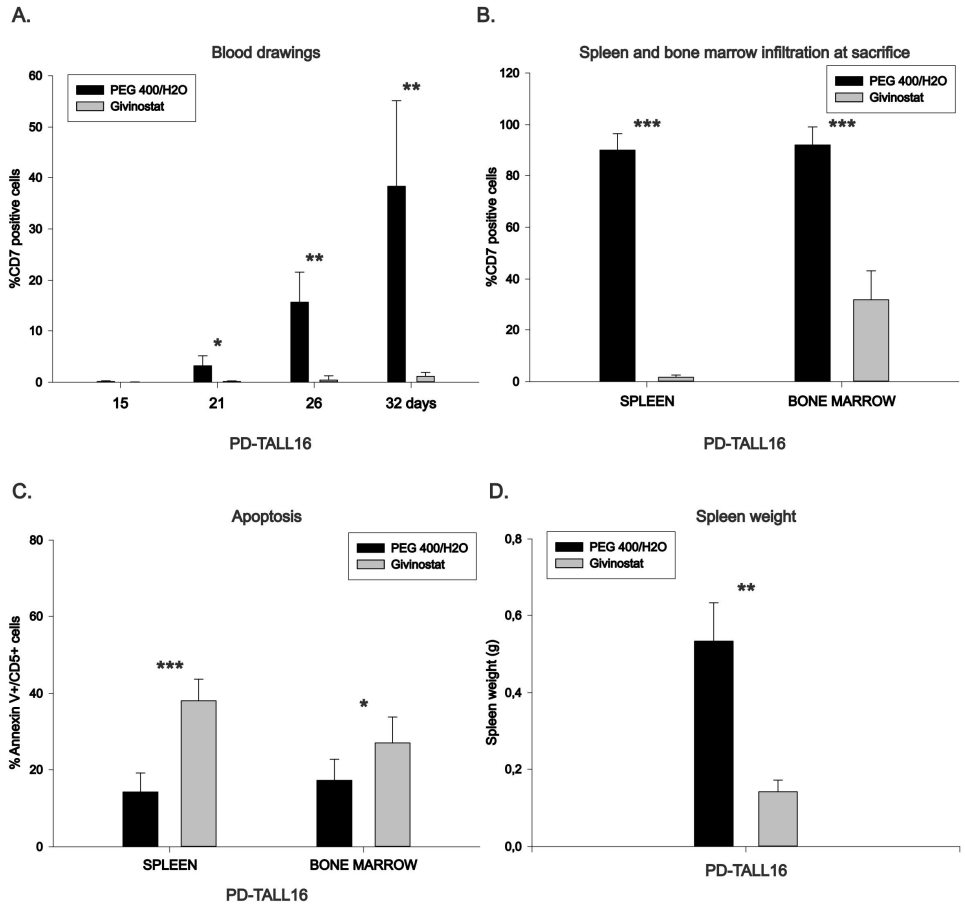


Fig. 17: Therapeutic effect of Givinostat in PD-TALL16 xenografts (TLX3). (n=6 mice/group) (* P<0.05, ** P<0.01, *** P <0.001).

Concerning PD-TALL12 (TAL-LMO), treatment with Givinostat determined a reduction over time of the percentage of CD7+ cells in the peripheral blood and a modest albeit significant reduction of leukemic cells infiltrating the spleen (from 80% to 30% of infiltrating cells). On the contrary, Givinostat did not decrease the level of infiltration of bone marrow, that was comparable between treated and control mice (Fig. 18 A and B). Evaluation of apoptosis in T-ALL cells harvested from the spleen of mice confirmed an increase in cell death (Fig. 18 C).

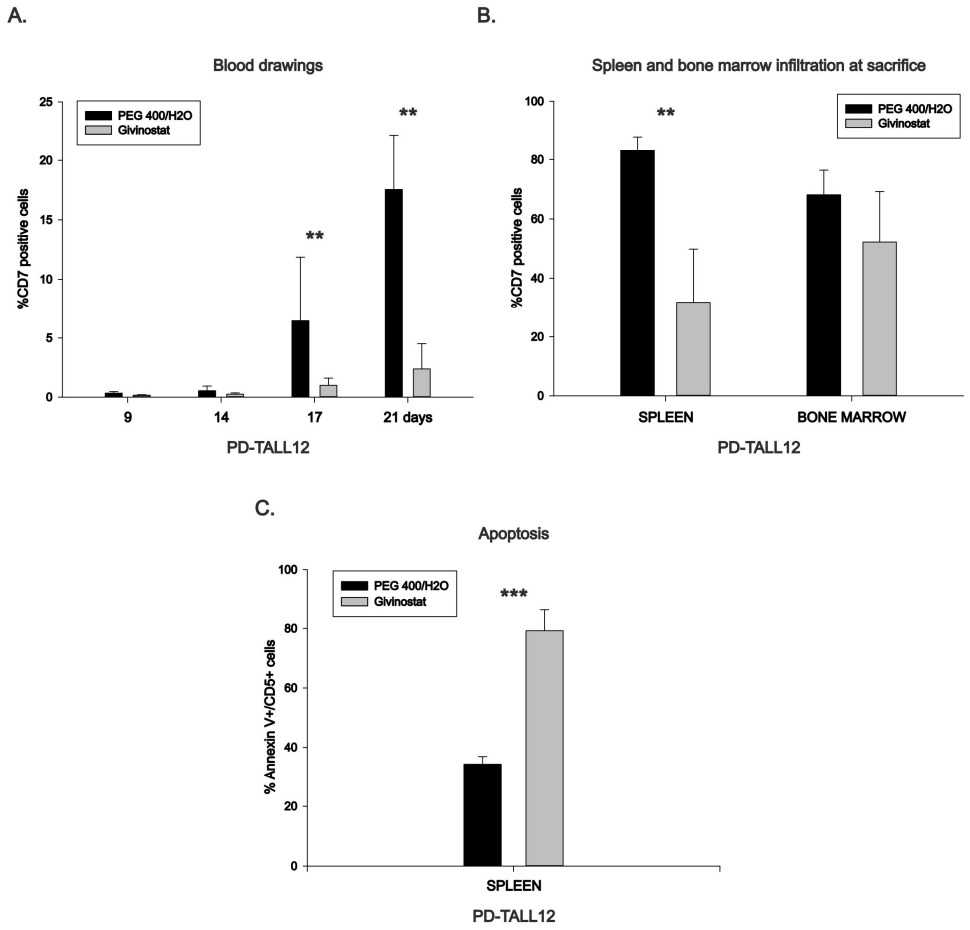


Fig. 18: Therapeutic effects of Givinostat in PD-TALL12 xenografts (TAL-LMO). (n=6 mice/group) (* P<0.05, ** P<0.01, *** P <0.001).

PD-TALL9, another leukaemia of the TAL-LMO subgroup, displayed less prominent effects compared to the other TAL-LMO xenograft PD-TALL12. On one hand, HDAC inhibition delayed T-ALL cells engraftment, as indicated by the significant reduction in the levels of CD7 positive cells in the blood of treated mice and by the decrease in spleen weight at sacrifice compared to controls (Fig. 19 A and D). On the other hand, there was no difference in the percentage of infiltrating and apoptotic cells in the spleen and the bone marrow of treated mice compared to controls (Fig. 19 B and C). Therefore, in PD-TALL9 Givinostat modestly delayed infiltration of spleen and bone marrow by leukemic cells, without affecting their viability.

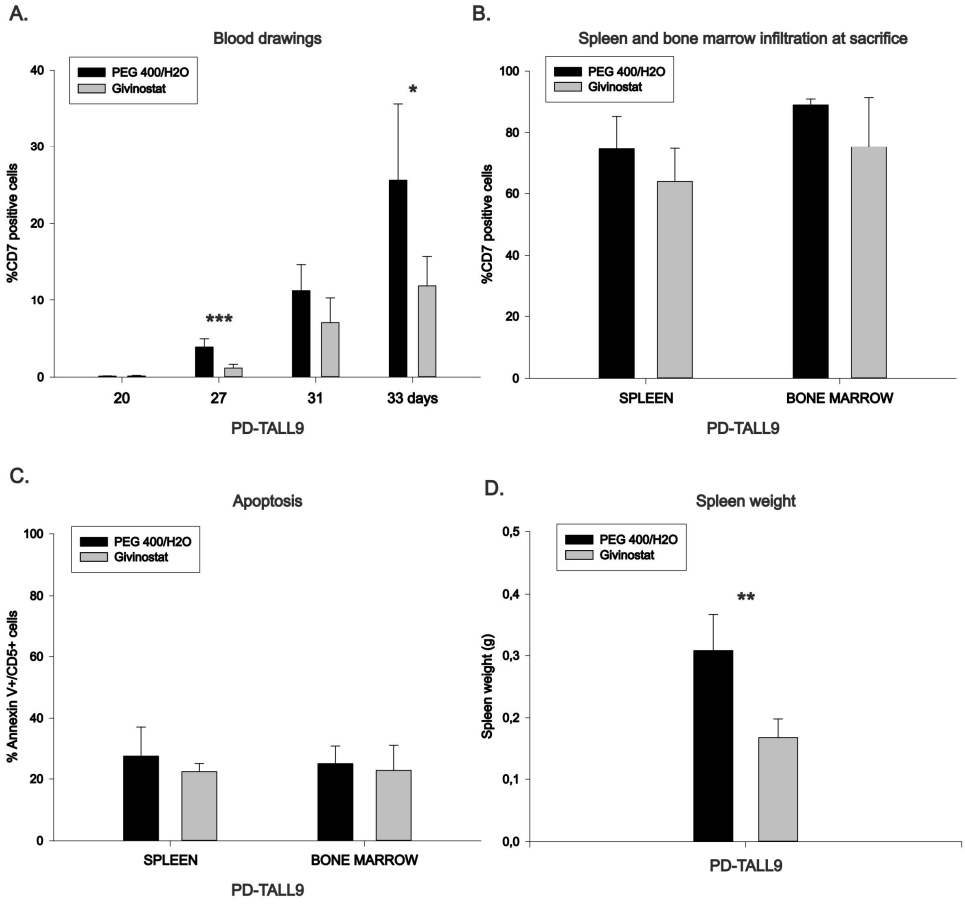
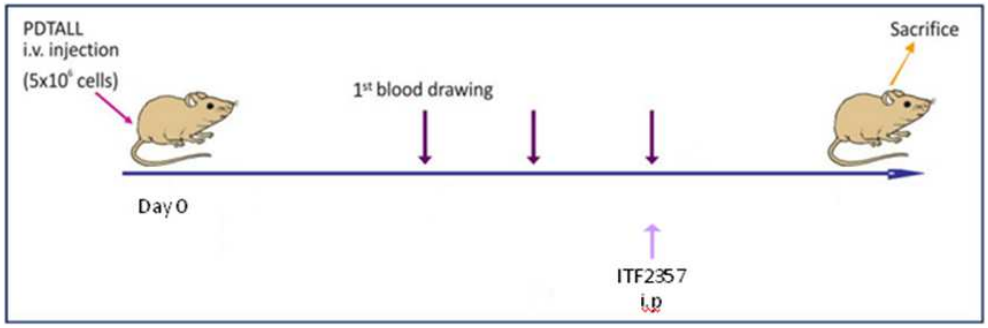


Fig.19: Therapeutic effects of Givinostat in PD-TALL9 xenografts (TAL-LMO). (n=5 mice/group) (* P<0.05, ** P<0.01, *** P <0.001).

In conclusion, down regulation of TAL1 protein induced by HDACi *in vitro* does not appear to correlate with therapeutic response to long-term treatment *in vivo* in TAL/LMO xenografts. On the other hand, TLX-driven xenografts PD-TALL8 and PD-TALL16 had better response to Givinostat, although neither TLX1 nor TLX3 protein levels were affected by HDAC inhibitors *in vitro*. These results suggest that TLX1/TLX3 T-ALL samples could be better responders to Givinostat.

4.9 Short term treatment *in vivo* using Givinostat

Since TLX 1 and 3 act as transcriptional repressor forming a complex with HDACs, we investigated whether HDACi could modulate TLX target genes expression. For this purpose, we performed short-term treatment in NOD/SCID mice with Givinostat. For this experiment, we used three primary xenografts used in the long-term treatment experiment. Briefly, T-ALL cells were injected i.v. in NOD/SCID mice at $5x10^6$ cells/mouse. Givinostat (25mg/kg) or PEG400/H_20 (vehicle) were administrated i.p once. The drug was administered when mice had full-blown leukemia, meaning percentage of blasts in the peripheral blood, bone marrow and spleen exceeding 10%, 70% and 80%, respectively (Fig. 20-22).
Mice were sacrificed 6 h after treatment. Spleen and bone marrow infiltration by T-ALL cells at sacrifice was very high and comparable between treated and untreated mice (Fig. 20-22). The experimental setting is represented in the following schematic drawing:



Cells were obtained from the spleen of the mice at sacrifice and mRNA levels of several TLX target genes were analysed by qRT-PCR. To confirm the efficacy of HDAC inhibition *in vivo*, we measured the acetylated form of α tubulin (Fig. 20-22).

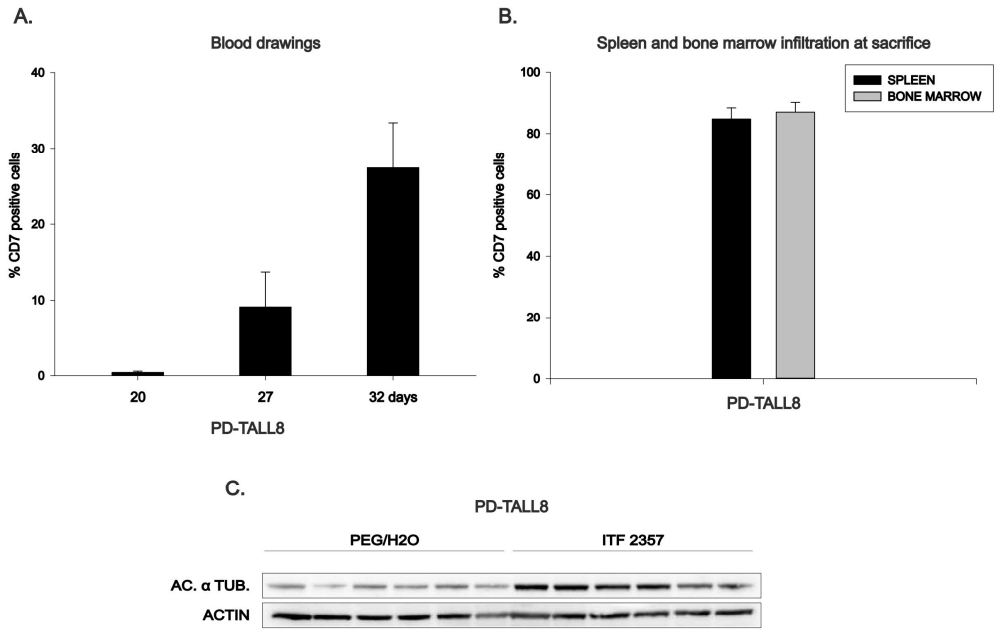


Fig. 20: Engraftment of PD-TALL8 cells in NOD/SCID mice. A) Leukaemia engraftment in NOD/SCID mice was evaluated by peripheral blood drawings analysis at day 20, 27 and 32 after T-ALL cells injection. **B)** Spleen and bone marrow were completely infiltrated at sacrifice 32 days after leukemic cells injection. **C)** Accumulation of acetylated α tubulin, was measured as a reed-out of HDAC activity inhibition by western blot analysis.

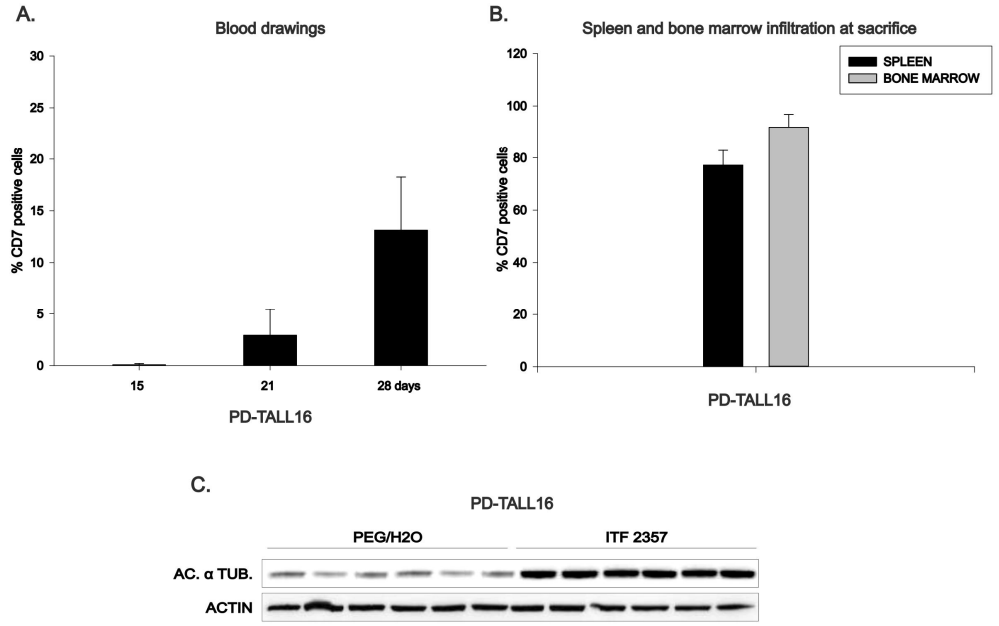


Fig. 21: Engraftment of PD-TALL16 cells in NOD/SCID mice

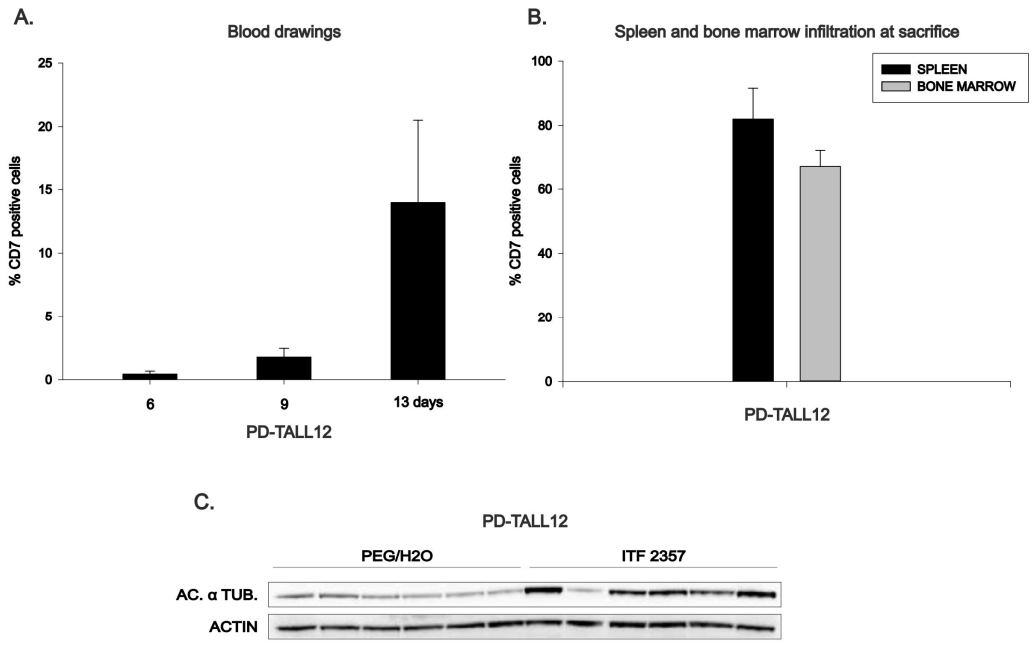


Fig. 22: Engraftment of PD-TALL12 cells in NOD/SCID mice

We analysed expression levels of a panel of TLX-related target genes including *ALDH1A1* (Rice et al., 2008), *RUNX1, GBP5, PTPN14, PLXD1* (Della Gatta et al., 2012) and *CCR7* (Rakowski et al., 2011). Moreover, we assessed the expression of several TAL1 and c-MYB target genes, including *TRIB2, STAT5A, BIM1* and *GATA2* (Lorenzo et al., 2011; Sanda et al., 2012; Waldron et al., 2012) and NOTCH target genes, including *CR2, DTX1* and *pTα*. Interestingly, expression of several TLX-target genes was modulated in treated mice compared to controls. In particular, the most up-regulated genes in all xenografts analysed were *ALDH1A1, GBP5* and *CCR7*, confirming the effect of Givinostat on TLX signalling. With regard to TAL1 and c-MYB-related target genes, *STAT5A* was reduced in PD-TALL12 and PD-TALL8, whereas *BIM1* was down-regulated in PD-TALL8. The NOTCH target genes most regulated *in vitro* (*CR2, DTX1* and *pTα*) did not display significant reductions in mRNA levels *in vivo* (Fig. 23).

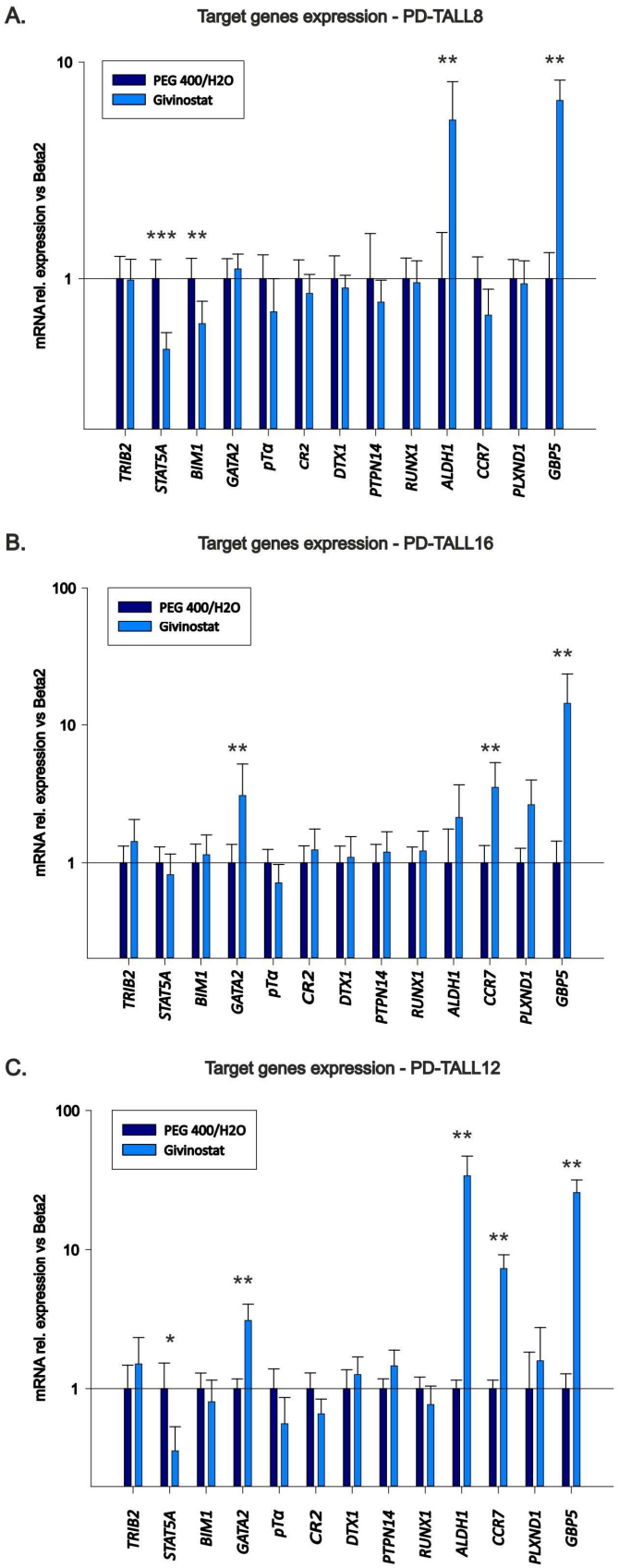


Fig. 23: Evaluation of TAL1, c-MYB, NOTCH and TLX target genes expression. T-ALL cells were recovered from mice spleen and mRNA expression of several target genes were assessed in PD-TALL8, PD-TALL16 and PD-TALL 12 (A, B and C) by qRT-PCR (n=6 mice/group) (* P<0.05, ** P<0.01, *** P <0.001).

5. DISCUSSION

In recent years, HDACi have emerged as candidate new drugs for cancer treatment. The therapeutic potential of HDACi stems from their higher cytotoxic activity against tumour cells compared with normal cells. Although numerous studies on HDACi have been published during the last decade, the mechanisms underlying their anti-tumour effects are still substantially unknown. In particular, the oncogenic pathways modulated by these compounds have not been fully elucidated. This lack of knowledge prevented so far identification of a subset of patients who might be responsive to these drugs.

The goal of my project was to investigate the impact of HDAC inhibitors on signalling pathways relevant in T-ALL *in vitro* and investigate whether this knowledge might predict therapeutic efficacy of these drugs in preclinical models of T-ALL.

The first step was to evaluate the pro-apoptotic and anti-proliferative effect of a canonical HDACi (TSA) *in vitro* in T-ALL cell lines and in a panel of primary T-ALL cells recovered from xenografts. TSA induced high levels of apoptosis in all samples, a finding which was somewhat at odds with our working hypothesis. This effect was not associated with the genetic subgroup of T-ALL, the NOTCH1 mutational status or other genetic features of xenografts. Unfortunately, primary cells from xenografts do not proliferate *in vitro*, thus preventing the possibility to measure anti-proliferative effects of TSA.

We next investigated the effect of HDACi on several transcription factors implied in T-ALL pathogenesis (TAL1, LMO2, TLX1, TLX3, c-MYB, NOTCH1 and NOTCH3). Although some recent studies reported the effects of HDACi on individual transcription factors (TAL1 and NOTCH3) (Cardoso et al., 2011; Palermo et al., 2012), this is the first comprehensive work which sets out to analyse the effect of HDACi on key signalling pathways in T-ALL. Our results disclosed regulation of several transcription factors by various mechanisms, including both transcriptional (in the case of TAL1 and c-MYB) and post-transcriptional (in the case of NOTCH) levels. Results were further validated by

measurements of expression levels of certain target genes of the various transcription factors. In the case of NOTCH, we unexpectedly observed TSA-mediated induction of HES1, one of the most important effectors of Notch signalling. However, when we checked HES1 protein levels we found them decreased after TSA treatment in both MOLT3 and DND 41 cells (data not shown). This observation could suggest the existence of compensatory circuits, in which low HES1 protein levels up-regulate HES1 transcription. Concerning TLX1 and TLX3, HDACi probably affect the repressor complex formation on TLX target genes promoters, blocking HDACs activity. Thus, protein levels were not altered by HDACi, but there was a modulation of the genes downstream TLX1 and 3, detected by qRT-PCR in short-term experiments *in vivo*, as shown in Fig. 23.

But are modulations of transcription factors essential to the pro-apoptotic activity of the drug? In the case of TAL1, its contribution to HDACi-induced apoptosis has been recently demonstrated in T-ALL by Cardoso et al. (Cardoso et al., 2011) To investigate this in the case of other transcription factors modulated by HDACi, we performed rescue experiments. We found that over-expression of the active forms of NOTCH1 and NOTCH3 prevents apoptosis upon TSA treatment. This result suggests a substantial contribution of NOTCH signalling in this phenomenon, although it should be noted that T-ALL cells bearing NOTCH1 activating mutations had similar levels of apoptosis as NOTCH1 wild-type cells. We are currently using a similar approach to investigate the role of c-MYB in HDACi-induced cell death, considering the involvement of this oncoprotein in T-ALL (Clappier et al., 2007; Lahortiga et al., 2007; Sarvaiya et al., 2012; Stenman et al., 2010).

Furthermore, we investigated the mechanism underlying the post-transcriptional regulation of NOTCH1 and NOTCH3 by HDACi. Interestingly, NOTCH1 and NOTCH3 down-regulation upon TSA treatment was rescued by a lysosome inhibitor, which suggests involvement of the endocytic pathway. This is in line with a previous work that demonstrated an increase in protein degradation of the Epidermal Growth Factor Receptor (EGFR) by the endocytic compartment following treatment with HDACi. In this study, HDACi increased microtubule acetylation and accelerated microtubule-dependent movement of EGFR-bearing

vesicles to the lysosome by an HDAC6-mediated mechanism (Gao et al., 2010). Alternatively, it could be that NOTCH1 and NOTCH3 are hyperacetylated following treatment with TSA and this might increase their turnover. These hypothesis, if confirmed, would indicate that the mechanism of action is more related to transcription factor rather than global hyperacetylation, as recently found in some experimental models (Christensen et al., 2014). With regard to NOTCH receptors and HDACi, Palermo et al. recently demonstrated down-regulation of NOTCH3 active ICD domain by TSA in MOLT3 cell line and in NOTCH3 transgenic mice (Palermo et al., 2012). In this paper, NOTCH3 ICD acetylation is unbalanced by HDAC inhibition, leading to increased ubiquitination and proteasome-dependent degradation. Our results on HDCAi-dependent NOTCH3 full length degradation by the lysosome add a novel mechanism of NOTCH3 full-length regulation. Furthermore, in our experiments the proteasome inhibitor MG132 further decreased NOTCH3 full-length protein levels. It will be interesting to test apoptosis following treatment with HDACi in combination with MG132, considering their additive negative effect on NOTCH3 levels. In this regard, previous papers reported synergistic effects of proteasome inhibitor Bortezomib and HDACi in haematological malignancies such as B-cell precursor ALL and CLL *in vitro* (Bastian et al., 2013; Dai et al., 2008).

To reinforce these findings we tested Givinostat (ITF 2357), a pan-HDACi now in clinical trials. Induction of apoptosis and inhibition of proliferation upon Givinostat treatment *in vitro* were comparable to TSA and, once again, similar levels of apoptosis were detected among the different xenografts. Moreover, Givinostat showed similar effects on transcription factors as TSA. Interestingly, we found that p16INK - a well-known regulator of the cell cycle (Romagosa et al., 2011) - was strongly up-regulated after Givinostat treatment. This result is in accordance with previous studies linking HDACi activity to changes in the expression of cell cycle regulatory genes (Fandy et al., 2005; Siavoshian et al., 2000) and is consistent with the anti-proliferative effect induced by HDACi in our *in vitro* studies. This finding also shows that the decrease in protein levels of various transcription factors was not due to a generalized negative effect on gene expression, as HDACi treatment clearly up-regulated the protein levels of this cell cycle inhibitor.

Supported by these *in vitro* results, we investigated the *in vivo* activity of Givinostat in a T-ALL mouse model developed in our laboratory. This systemic model of T-ALL reproduces the natural history of this tumour and it is an useful tool to test the therapeutic activity of new drugs. We adopted short-term and long-term treatments to analyse the biochemical and therapeutic effects of Givinostat, respectively.

Analysis of TLX-related target genes revealed up-modulation of *ALDH1A1*, *GBP5*, *CCR7 in vivo* following Givinostat administration. The modulation of these target genes at an early time point from drug administration suggests that Givinostat blocks HDACs activity in the TLX-repressor complex. We also detected modulation of *STAT5A* and *BIM1*, two TAL1 and c-MYB target genes, respectively, although these effects were not shared by all the xenografts tested. On the contrary, NOTCH target genes remained invariably stable upon Givinostat treatment. Since NOTCH receptors are hypothetically down-regulated by HDACi through post-transcriptional mechanisms, it is possible that the relatively short time from drug administration to sacrifice of the mice (6 h) was not sufficient to reduce expression of NOTCH target genes.

Finally, we asked whether primary leukaemias belonging to different T-ALL subgroups could respond differentially to long term treatment with Givinostat *in vivo*. In our experiments, TLX-driven xenografts including PD-TALL8 (TLX1) and PD-TALL16 (TLX3) were the best responders to Givinostat. Anti-tumour effects were predominant in the spleen compared with the bone marrow, perhaps due to some features of the BM microenvironment, such as hypoxia, which may protect T-ALL cells from drug-induced apoptosis. The TAL-LMO xenografts (PD-TALL12 and PD-TALL9) had minor therapeutic responses to Givinostat.

Since TLX1/TLX3 are transcriptional repressors of differentiation through a molecular complex involving also HDAC (Dadi et al., 2012; Sun et al., 2007), ongoing experiments are aimed at investigating whether Givinostat may primarily act by restoring cell differentiation.

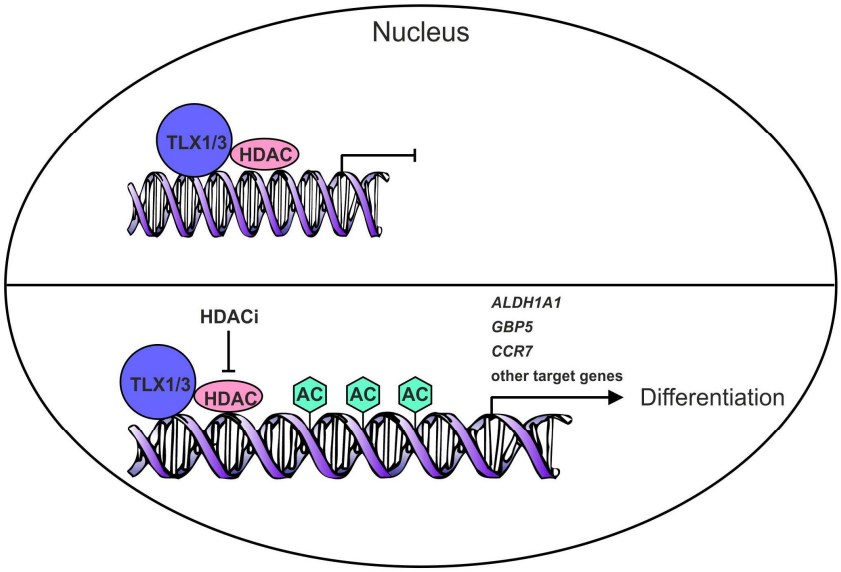


Fig. 24: Putative mechanism of action of HDACi in TLX-driven T-ALL xenografts. In normal conditions, TLX1/3 recruit HDACs and form a silencing complex on target genes promoter, leading to transcriptional repression (top). Upon HDACi treatment, HDACs activity is impaired and histones are hyperacetylated. Chromatin expansion is associated with increased expression of TLX target genes that induce differentiation of T-ALL cells.

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