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Mechanism of action of Histone Deacetylase Inhibitor - Givinostat in Chronic Myeloproliferative Neoplasm

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Chapter 1

Introduction

Chemical modifications of DNA and histone proteins form a complex regulatory network that modulates chromatin structure and genome function[1,2]. The core histones are predominantly globular except for their N-terminal ''tails,'' which are unstructured[3,4].

During recent years, considerable research efforts have focused on potentially reversible alterations in chromatin structure, which modulate gene expression during malignant transformation.

For these reasons, in the last years the interest of histone deacetylase inhibitors have gained considerable interest for clinical use in a variety of pathological conditions, and the study of its effect on tumor cells indicates that this class of drugs may become useful therapeutic agents in oncology.

1.1 Chromatin structure and Remodeling

1.1.1 Chromatin structure

The eukaryotic genome is assembled as a nucleoprotein complex known as chromatin which is the state of DNA, when it is packaged within the cell[1]. The basic structure of chromatin is the nucleosome core particle, which is formed by ~145-147 base pairs (bp) of DNA that are wrapped in 1.67 left-handed superhelical turns around a core histone octamer. The histone octamer is made up of two copies of each histone proteins: H3-H4 tetramer and two H2A-H2B dimers[5,6]. Core histones within the nucleosome contain a globular domain which mediates histone-histone interaction and also bear a highly dynamic N-terminal tail of around 20-35 residues rich in basic aminoacids. These tails extend from the nucleomes's surface, protruding from the nucleosome[7]. All of these regions, particularly the positively charged N-terminal tails protruding from the DNA helix, are sites for a variety of covalent modifications. Indeed there are at least eight distinct types of modification found on histones, acetylation, methylation, phosphorylation, ubiquitination, biotinylation, sumoylation, ADP ribosylation, deimination, and proline isomerization. These dynamic alterations modulate interactions between DNA, histones, multiprotein chromatin

remodeling complexes, and transcription factors, thereby enhancing or repressing gene expression[8].

The nucleosome is stabilized by a multitude of protein–protein interactions within the histone octamer and by numerous electrostatic and hydrogen bonds between protein and DNA over its entire length. The vast majority of DNA–histone interactions are between structured regions of the histones and DNA, whereas the flexible histone tails (the sites of most post-translational modification) extend away from nucleosomal DNA and are mainly involved in interaction with neighbouring nucleosomes or with nuclear factors, and play an important role in folding of nucleosomal arrays into higher order chromatin structure[9,10].

The packaging of DNA by histones into chromatin is a key regulator of transcription and other nuclear processes that involve DNA. The structure of the first level of DNA organisation, the linear arrangement of features such as nucleosome on DNA, has been determined to high resolution. Chromatin fibers are capable of condensing into multiple 'higher order' structures, now better thought of as secondary and tertiary chromatin structures. The next level of DNA compaction, is highly condensed, 30 nm diameter secondary structure formed by repetitive folding of adjacent nucleosomes (the '30 nm fiber') and is the most intensely studied chromatin structure, after the nucleosome. The tertiary chromatin structure is formed by interactions between secondary structures, like thicker fibers seen in nuclei and postulated to be composed of bundles of 30 nm fibers[5].

It is widely recognized that the compact and folded higher order structure of chromatin represses such DNA-dependent

activities as transcription, replication and repair, and that decompaction of chromatin is instead a prerequisite for these processes[11].

While three distinct levels of chromatin structure organization have been outlined above, in reality the scale of chromatin condensation is rather dynamic and structurally heterogeneous, allowing numerous nuclear events such as transcription, repair, replication and recombination to occur. Indeed chromatin is organized into domains, such as euchromatin and heterochromatin, which have different chromosomal architecture, transcriptional activity and replication timing[4].

Heterochromatin is representative of a compact or inaccessible chromatin structure inherent to transcriptionally inactive regions of DNA and is present at the chromosome centromere and telomeric regions. Euchromatin, on the other hand, is typical of uncondensed chromatin lacking histone H1, is amenable to gene activation and exists in gene rich environments. ATP-dependent chromatin remodeling complexes and histone modifying enzymes are two of the factors known to modulate the structure of chromatin, which will be discussed in further detail below[13].

1.1.2 Epigenetic and Histone Modifying Enzymes

Epigenetics is the study of heritable changes in gene expression that do not involve changes in the DNA sequence level. The principal epigenetic mechanisms by which tissue-specific gene-expression patterns and global gene silencing are established and maintained are chromatin modification and chromatin remodeling[6].

Studies of the molecular mechanisms that mediate epigenetic regulation include DNA acetylation and chromatin/histone modifications, and both mechanisms act in concert to provide stable and heritable silencing in higher eukaryotic genomes. Histone modifiers encompass a broad category of enzymes responsible for introducing post-translational covalent modifications onto histone proteins. Whereas the majority of nucleosomes in the cell are composed of the same four types of core histones, a high diversity in the histone/nucleosome structures is generated by a variety of posttranslational modifications, such as acetylation, phosphorylation, methylation, and ubiquitination[12,14]. Some modifications, including acetylation and phosphorylation, are reversible and dynamic and are often associated with inducible expression of individual genes. Other modifications, such as methylation, which occur on specific sites on the histones, are more stable and involved in the long-term maintenance of the expression status of entire regions of the genome[15].

Although it has been known for many years that histones in eukaryotes are modified by acetylation, it is only in the past decade that the role of histone acetylation in transcription regulation has been identified. Of all the histone modifications, acetylation is the most studied, and is part of the thesis study. The acetylation of lysine residues at core histone N-terminal tails weakens the interactions between core histones and DNA, which in turn destabilizes the nucleosomal structure and ultimately facilitates binding of transcription factors to DNA. Hyperacetylation is generally thought to favor gene expression, whereas deacetylation by histone deacetylases usually brings about the silencing of the genes (Figure

1). Therefore, histone modifications modulate chromatin organization and regulate many nuclear processes, including transcription[16].

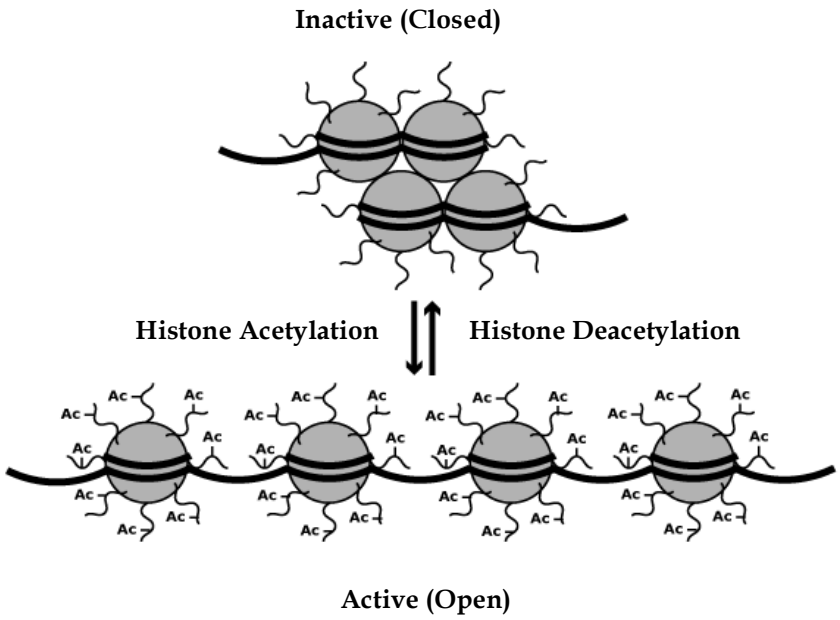

Figure 1. The states of chromatin and regulation of gene expression Histone proteins have N-terminal tails that extend out from the nucleosome core. Histone acetylation involves the attachment of acetyl groups to lysine residues in the N-terminal tails of histone proteins. It is believed that the acetylation of lysine (changing a positively charged residue to a negatively charged residue) decreases the affinity for histones for DNA (and possibly histones for other histones), thereby making DNA more accessible for transcription. The opposite reaction (deacetylation) removes acetyl groups from lysine residues in the N-terminal tails of histone proteins. (*Adapted from University of California; Genes and Genomes; 2007 Jul; http://missinglink.ucsf.edu/lm/genes_and_genomes/acetylation.html)*

Histone acetylation is a dynamic process controlled by the antagonistic actions of two large families of enzymes, the histone acetyltransferases (HATs) and the histone deacetylases (HDACs). The balance between the actions of these enzymes serves as a key regulatory mechanism for gene expression and governs numerous developmental processes and disease states.

In the following section histone modifiers will be discussed, with particular emphasis on these HATs and HDACs enzymes.

1.1.2.1. Histone Acetyltransferase

There is a growing body of evidence supporting the notion that acetylation, like phosphorylation, is an important regulatory protein modification. HATs are increasingly being recognized as modifiers of both histones and nonhistone proteins. HATs function enzymatically by transferring an acetyl group from acetyl-coenzyme A (acetyl-CoA) to an ε-amino group of certain lysine side chains within a histone's basic N-terminal tail region. Lysine acetylation neutralizes part of a histone tail region's positive charge, breaking up the electrostatic interactions between the negatively charged phosphodiester DNA backbone and the positively charged amino-terminal histone tails, resulting in weakened histone-DNA or nucleosome-nucleosome interactions[17]. The theory follows that consequent weakening of histone-DNA electrostatic interactions leads to a more relaxed chromatin structure that is open to transcription factor target site binding and acetylated chromatin prefers to associate with transcription factors.

HATs also acetylate lysine residues within transcription-related proteins in addition to histones. HAT proteins form multiple complexes and are recruited to chromatin where they acetylate histones or transcription factors[18].

HATs have traditionally been divided into two types, A and B, that are different in origin and function. Type A HATs are located in the nucleus, and acetylate nucleosomal histones as well as other chromatin associated proteins; as such, these HATs directly modulate gene expression. In contrast, type B HATs are localized in the cytoplasm, and acetylate newly synthesized histones, thus facilitating their transport into the nucleus and subsequent association with newly synthesized DNA[19,20].

Subsequently, a flurry of studies led to the discovery of a large number of HATs enzymes, such as GNAT superfamily (PCAF, GCN5), MYST family proteins including MOZ, nuclear receptor coactivators, and p300/CBP, the latter had previously been identified as a transcriptional coactivator. Interestingly, while histones were the first elucidated targets of HATs, a number of non-histone proteins have been uncovered as HAT substrates. Aptly, the term 'acetylome' has recently been coined, cataloging all HAT targets. The transcription factor p53 was the first non-histone protein to be identified as a HAT substrate[21]. Moreover beyond affecting DNA binding and protein stability, HAT facilitate acetylation of non-histone proteins, and have also been documented to influence protein-protein interactions, protein dimerization and protein localization (Figure 2).

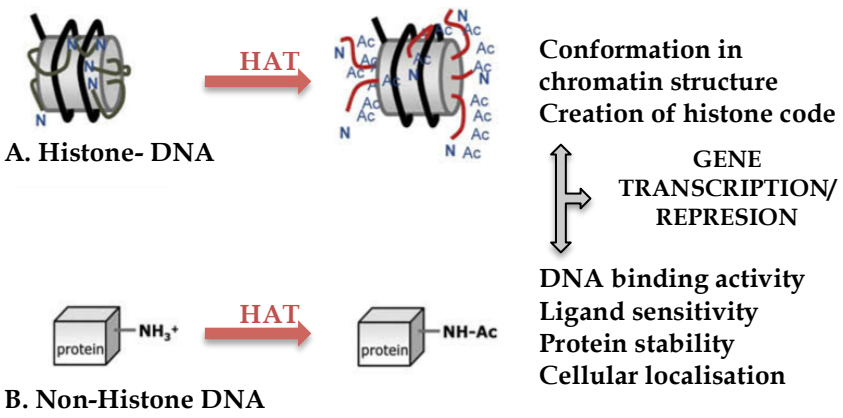

Figure 2. HAT mediated transcriptional control. (A), Histone-related pathway: HATs acetylate histones resulting in reduced chromatin compaction. (B) Non-histone related pathway: HDACs directly interfere with non- histone protein targets, including transcription factors, nuclear hormone receptors, nuclear import factors, structural proteins and adhesion proteins. Acetylation of non-histone proteins might affect diverse aspects of their protein physiology, resulting in either decreased or increased activity of the target protein. (*Adapted from Sarah Snykers; Journal of Hepatology; 2009 Jul; 51(1):187-211)*

Enzymes, which specifically acetylate nonhistone protein, are yet to be discovered. However it has been established in vitro and in vivo that nonhistone proteins are the bonafide substrates of HAT. Based on the cellular functions, nonhistone substrates for HATs and HDACs can be classified under the following categories: (a) General transcription factors, (b) Non-histone chromatin associated protein, (c) Nuclear receptor coactivators, and (d) Transcriptional activators or repressors[22,23].

a) General Transcription Factors. P300/CBP, PCAF and TAFII250, the components of basal machinery TFIIE, TFIIF. More specifically RAP74 and RAP30 subunits of TFIIF and subunit of TFIIE get acetylated. In vitro DNA transcription shows no specific effect as a result of the acetylation of these factors, the effect of acetylation on the chromatin context has not been verified.

b) Nonhistone Chromatin Associated Protein. High mobility group proteins are a heterogeneous class of non-histone chromosomal protein in eukaryotic organisms. They are among the most abundant and ubiquitous nonhistone proteins in the nucleus.

c) Nuclear receptor coactivators. Nuclear receptors regulate gene expression by their association with HAT or HDAC complexes. Hormones induce dramatic hyperacetylation at endogenous target genes by p300/CBP. This hyperacetylation is transient and coincides with attenuation of hormone induced gene activation.

d) Transcriptional activators or repressors. Activators are sequence-specific DNA binding proteins, which also interact with the transcription machinery directly or through coactivators. Recent evidence suggests that activators are the key molecules to recruit HATs and chromatin remodelling machineries to specific promoters that promote preinitiation complex assembly. In this process they may get acetylated along with the promoter proximal histones. In the following section we shall discuss the effect of acetylation, and subsequently deacetylation, on certain transcriptional activators that are directly correlated with transcriptional regulation.

Functional consequences of the acetylation of these proteins non-histones have been summarized in Table 1.

Type of protein	HAT	Functional consequence of acetylation
DNA binding transcription factors		
p53	p300/CBP, PCAF	Enhanced DNA binding.
C-Myb	p300/CBP, GCN5	Enhanced DNA binding (promotes cell proliferation and differentiation).
MyoD	p300/CBP, PCAF	Increased DNA binding (to transactivate muscle promoters).
E2F	PCAF, p300/CBP	Enhanced DNA binding affinity (to transactivate E2F responsive promotor).
GATA-1	p300/CBP	Enhanced DNA binding activity (stimulation of GATA-1 dependent transcription).
EKLF	p300/CBP	Enhanced globin gene expression (inhibitory domain modified).
Non-histone chromatin associated proteins		
HMGB1, HMGB2	CBP	Acetylation enhances their ability to bend DNA.
HMGN1, HMGN2	p300 p300/PCAF	Alters their interaction with chromatin (weakens their ability to bind to nucleosomal cores).
HMGA	P300/CBP	Enhanceosome disruption (transcription repression).
	PCAF	Enhanceosome assembly (transcription activation).
General transcription factors		
TFIIB	Autoacetylated	Stabilizes interaction between TFIIB and TFIIF and stimulates transcription.
TFIIEβ	PCAF, p300/CBP, TAFII50	Unknown
TFIIF	p300, PCAF	Unknown
Viral proteins		
Tat	PCAF	Enhanced transcription (increased CDK9. binding).
	p300/CBP	Enhanced transcription (decreased affinity between Tat and TAR RNA).

Table 1. Effects of acetylation of the various groups of non-histone proteins. (*From Chandrima Das; IUBMB Life; 2005 Mar; 57(3):137-49)*

For the focus of our studies, it is important to emphasize here the presence of C-MYB among HAT targets. Indeed C-MYB belongs to a family of transcription factors which regulate differentiation and proliferation in immature hematopoietic and lymphoid cells. Improper regulation of any of these processes leads to leukemogenesis. The C-MYB proto-oncogene product (C-MYB) is a

sequence-specific DNA-binding protein that functions as a transcriptional activator, which has multiple cellular roles during hematopoiesis. Upon induction by cytokine, C-MYB expressing normal cells favor self-renewal. However when the endogeneous activity of this protein is inhibited, differentiation and apoptosis are favored over self- renewal. Further, inhibition of C-MYB activity also causes reduced cell survival and proliferation[22]. Function of C-MYB protein is also acetylation dependent. p300 acetylates the conserved C-terminal domain of C-MYB which stimulates its DNA binding activity. Cotransfection of C-MYB along with p300, leads to enhanced in vivo gene expression of a C-MYB dependent promoter, as assessed by reporter gene assays. Though acetylation of C-MYB by GCN5 is reported in vitro, CBP can also acetylate C-MYB in vivo. CBP mediated acetylation increases the trans-activating capacity of C-MYB. Therefore, acetylation of C-MYB by CBP/p300 has two functional consequences for C-MYB activity: increased DNA binding activity and increased activation potential[24].

1.1.2.2. Histone Deacetylase

In opposition to HAT activity, histone deacetylase (HDAC) are part of a vast family of enzymes that have crucial roles in numerous biological processes, largely through their repressive influence on transcription, catalyzing the removal of acetyl moieties on histone residues and other non-histone proteins[25,26]. Conversely to acetylated histones that often act as transcriptional activators, histone deacetylation favours transcriptional repression by allowing for chromatin compaction. HDACs have been implicated as regulators of

gene expression, especially during cell differentiation and development, and do not bind directly to DNA but seem to be recruited to specific promoters through their interaction with DNA sequence-specific transcription factors[27].

Therefore, it is assumed that the balance between HAT and HDAC activity dictates the overall histone acetylation status and, accordingly, the level of transcriptional competence[28].

HDACs are currently divided into four classes based on phylogenetic and functional criteria Table 2:

HDACs

Class	Enzymes	Zn2+ Dependent	Localization	Expression
I	HDAC 1, 2, 3 and 8	YES	Nucleus	Ubiquitous
IIa	HDAC 4, 5, 7 and 9	YES	Nucleus and Cytoplasm	Tissue specific
IIb	HDAC 6 and 10	YES	Cytoplasm	Tissue specific
III	Sirtuins 1-7	NO	Variable	Variable
IV	HDAC 11	YES	Nucleus and Cytoplasm	Ubiquitous

Table 2. The four classes of HDACs. Classes I, II, and IV all contain a zinc (Zn) molecule in their active site and are inhibited by the pan-HDAC inhibitors. The seven different class III HDACs (sirtuins), are homologous to the yeast Sir2, do not contain Zn in the active site, and are not inhibited by any current HDAC inihibitors. *(Adapted from Andrew A. Lane; J Clin Oncol; 2009 Nov 10; 27(32):5459-688)*

Class I consists of HDAC1, 2, 3 and 8; which share homology with Rpd3, a founding member from budding yeast. These HDACs are expressed ubiquitously, localized predominantly in the nucleus and display high enzymatic activity toward histone substrates. They

possess relatively simple structures, consisting of the conserved deacetylase domain with short amino and carboxy-terminal extensions[25,29].

Class II can be divided into two subclasses, Class IIa, which contains HDAC4, HDAC5, HDAC7 and HDAC9, and Class IIb, formed by HDAC6 and HDAC10.

The Class IIa members, in contrast to other HDACs, show relatively restricted expression patterns, with HDAC4 being highly expressed in the brain and growth plates of the skeleton, HDAC5 and HDAC9 in muscles, heart and brain, and HDAC7 in endothelial cells and thymocytes (T-cell precursors derived from the thymus). In the Class IIb family, the HDAC6 is the main cytoplasmic deacetylase in mammalian cells, whereas HDAC10 is principally expressed in the liver, spleen and kidney. Among the targets directly deacetylated by HDAC6 are cytoskeletal proteins such as α-tubulin and cortactin, transmembrane proteins such as the interferon receptor IFNαR, and chaperones. HDAC6 is distinct from all other HDACs, as it harbours two deacetylase domains and a C-terminal zinc finger.

Class I, II and class IV HDACs are zinc-dependent enzymes containing catalytic pockets that can be inhibited by zinc chelating compounds such as hydroxamic acid.

Class III consists of members of the sirtuin family of HDACs; they are highly conserved, both functionally and structurally and play complex and important roles in the deregulation of metabolism[25].

There are seven members in humans, divided into four classes, and evolutionarily conserved orthologues can be found in most forms of life, including both eukaryotes and prokaryotes. They

are structurally similar to yeast SirT2, and require NAD+ as a cofactor for enzymatic activity, and are virtually unaffected by all HDAC inhibitors in current development. These proteins have not been extensively studied in mammalian systems, although Sir2 has recently been shown to deacetylate p53 at the carboxy terminus and inhibit p53-mediated transcriptional activation and apoptosis[30].

Class IV is represented by HDAC11, which is expressed in the brain, heart, muscle, kidney and testis, but little is known about its function, and it shows conserved residues within the catalytic domain which are shared with class I and class II HDACs

While HDACs have been extensively implicated as transcriptional repressors, a number of recent reports suggest that HDAC activity may be required for gene activation in some cases, and HDAC activity has also been shown to be necessary for the transcription of many interferon stimulated response genes. Therefore, in addition to acting as transcriptional co-repressors, HDACs appear to play a role in the transcriptional activation of particular genes[14].

With respect to non-histone substrates for HDACs, these too can be classified under four categories as has been described above, and functional consequences of the deacetylation of these proteins have been summarized in Table 3[31].

Type of protein	HDAC	Functional consequences of deacetylation
DNA binding transcription factors		
p53	HDAC1,Sir2	Downregulates p53 transcriptional activity by reducing its DNA binding ability. Apoptosis inducing activity also reduced.
MyoD	HDAC1	Inhibits its ability to convert naïve Fibroblasts to muscle cells, i.e., impedes cell differentiation.
E2F	HDAC1	Repression of E2F target promoters.
GATA-1	HDAC5	Suppresses transcriptional potential of GATA-1.
Viral proteins		
Tat	HDAC1	Inhibits the transcription from HIV promotor. YY1 recruits HDAC (expression from LTR downregulated).

Table 3. Effects of deacetylation of the various groups of non-histone proteins. (*From Chandrima Das; IUBMB Life; 2005 Mar; 57(3):137-49)*

1.1.3 Chromatin Modification in Cancer and Histone

Changes in growth and differentiation leading to malignancy

seem to occur by alterations in transcriptional control and gene silencing. It is becoming increasingly apparent that imbalances of both DNA methylation and histone acetylation may play an important role in cancer development and progression[31;32]. Therefore a delicate balance between histone acetylation and histone deacetylation is essential to carry out accurate gene regulatory events within the cell, and disruption of HAT and HDAC function is associated with the development of cancer, and malignant cells target chromatin-remodeling pathways as a means of disrupting transcriptional regulation[33]. Of the various hypotheses describing deregulation mechanisms, the following are most frequently cited:

1) Disordered hyperacetylation could activate promoters that are normally repressed, leading to inappropriate expression of proteins.
2) Abnormally decreased acetylation levels of promoter regions could repress the expression of genes necessary for a certain phenotype.
3) Mistargeted or aberrant recruitment of HAT/HDAC activity could act as a pathological trigger.

Inactivation of HAT activity due to gene mutations or the inhibitory action of viral proteins is associated with cancer. For example, functional mutations in one *CBP* allele is associated with Rubinstein–Taybi syndrome, a condition that predisposes to cancer, and p300 missense mutations and loss of heterozygosity at the p300 locus are associated with colorectal and breast cancers, and glioblastomas[34-36]. These interactions can antagonize the expression of cellular genes that are normally activated by p300, either by directly inhibiting HAT activity or by blocking the interaction of the

coactivators with DNA-binding proteins or basal transcription factors.

Even though no direct alterations in HDAC genes have been demonstrated in cancer, the association of HDACs with various oncogenes and tumor suppressor genes is now well established, as the potential for HDAC involvement in tumorigenesis. For example, HDAC1, HDAC2 and HDAC3 have been reported to be overexpressed in colon cancer, HDAC1 and HDAC2 are upregulated in gastric tumors, and HDAC1 has increased expression levels in hormone refractory prostate carcinoma. Additionally, systematic investigations of the function of all HDAC family members in a given tumor model are lacking and this information may be of importan clinical relevance.

Below in this section, we review the expression of the HDACs in cancer and our current knowledge on the genetics and function of the classical HDAC family members 1–11 in cancer Table 4.

Just as few examples, essential function of HDAC1 in proliferation control and p21 and p27 CDK inhibitor repression has been described in mouse embryonic stem cells[37,38]. In cancer cells, several studies have similarly found an important function of HDAC1 in controlling cell proliferation[39]. Knockdown of HDAC1 results in arrest either at the G1 phase of the cell cycle or at the G2/M transition, causes loss of mitotic cells, cell growth inhibition, and an increase in the percentage of apoptotic cells in osteosarcoma and breast cancer cells. On the contrary, HDAC2 knockdown showed no such effects in these cells. In addition to controlling cell cycle and apoptosis, HDAC1 might also be involved in multi-drug resistance[40,41]. HDAC1 was overexpressed in chemotherapy resistant

neuroblastoma cells in vitro and siRNA knock-down sensitized cells for etoposide treatment[42,43].

HDAC2 knockdown in cervical cancer cells induced a differentiated phenotype as well as increase in apoptosis associated with increased p21Cip1/WAF1 expression that was independent of p53. In breast cancer cells, HDAC2 knockdown increases the functional DNA binding activity of p53 associated with inhibition of proliferation and induction of cellular senescence[44,45].

On the other hand a fraction of diffuse large-B cell lymphomas shows high expression levels of the transcription factor, B-cell lymphoma 6 (*BCL6*). Upon hypoacetylation of BCL6 by HDAC2, the transcription of BCL6-responsive genes is suppressed, including that of cyclin dependent kinase inhibitor p21WAF1[46,47].

	HDAC family member	Expression in tumor tissues	Function in cancer cells
class I	HDAC1	*gastric cancer*: elevated expression, associated with nodal spread and poor prognosis; *pancreatic cancer:* expression associated with de-differentiation, enhanced proliferation and poor prognosis; *colorectal cancer*: increased expression associated with poor prognosis; *prostate cancer*: increased in high grade, hormone refractory cancers; *hepatocellular carcinoma*: high expression associated with portal vein invasion, poor differentiation, advanced TNM stage	*cervical cancer cells*: HDAC1 knockdown results in inhibition of proliferation and induction of autophagy; *osteosarcoma and breast cancer cells*: knockdown causes cell cycle arrest, growth inhibition, apoptosis; *colon cancer cells*: knockdown suppresses growth; *prostate cancer:* overexpression increases proliferation and de-differentiation; *neuroblastoma cells:* knockdown sensitizes for chemotherapy; *CLL cells*: knockdown sensitizes for TRAIL-apoptosis
	HDAC2	*colorectal cancer*: upregulation in polyps, associated with poor prognosis; *cervical carcinoma*: high expression in dysplasia; *gastric and prostate cancer*: increased expression associated with advanced stage and poor prognosis	*cervical cancer cells:* HDAC2 knockdown results in differentiation, apoptosis and p53 independent p21 expression; *breast cancer cells:* increased p53 activity, inhibition of proliferation, induction of senescence, induction of apoptosis; *colon cancer cells:* knockdown causes growth arrest; *neuroblastoma cells*: knockdown induces apoptosis; genetic HDAC2 mutation reduces intestinal tumor development in APC mice in vivo; *CLL cells*: knockdown sensitizes for TRAIL-apoptosis
	HDAC3	*gastric, prostate, colorectal cancers*: high expression associated with poor prognosis (together with HDAC1 and 2)	APL cells: HDAC3 associated with PML-RARa fusion protein, knockdown induces differentiation genes; *AML:* AML-1-ETO binds HDAC3 (and HDACs 1, 2), disrupts cell cycle
	HDAC8	childhood *neuroblastoma:* high HDAC8 expression significantly correlates with advanced stage disease, clinical and genetic risk factors and poor long term survival	*neuroblastoma cells*: HDAC8 knockdown induces differentiation, cell cycle arrest and inhibits clonogenic growth; *lung, colon, cervical cancer cells*: knockdown of HDAC8 reduces proliferation
class II A	HDAC4	*breast cancer:* upregulation compared with renal, bladder, colorectal cancer	*APL cells*: HDAC4 interacts with PLZF-RARa fusion protein, represses differentiation genes; renal carcinoma cells: knockdown inhibits expression and functional activity of HIF-1a
	HDAC5	*colorectal cancer:* upregulation compared with renal, bladder, breats cancer	*erythroleukemia*: HDAC5 shuttles from nucleus to cytoplasm upon differentiation, interacts with GATA-1
	HDAC7	*colorectal cancer*: high expression compared with bladder, renal, breast cancer tissues	*endothelial cells:* HDAC7 silencing alters morphology, migration and tube-forming capacity
	HDAC9	nd	nd
class II B	HDAC6	*oral squamous cell cancer*: high expression, increased in advanced stage; *breast cancer*: high expression correlates with response to endocrine treatment, inverse correlation of expression with survival and tumor size	Targeted inhibition of HDAC6 leads to acetylation of HSP90 and disruption of its chaperone function, resulting in depletion of pro-growth and pro-survival client proteins including the Bcr-Abl oncoprotein in *K562 leukemic* cells; HDAC6 targeting blocks EGF induced nuclear translocation of ß-catenin and c-myc expression in *colon carcinoma* cells; knockdown of HDAC6 causes donwregulation of HIF-1a, VEGFR1/2; HDAC6 invovled in TGFb induced epithelial-mesenchymal transition of *lung carcinoma* cells
	HDAC10	nd	Knockdown of HDAC10 downregulates VEGFR
class IV	HDAC11	nd	nd

Abbreviations: CLL, chronic lymphatic leukemia; APL, acute promyelocytic leukemia; AML, acute myeloid leukemia; RARa, retinoic acid receptor alpha; CML, chronic myeloid leukemia; nd, no data

Table 4. HDACs in cancer: Expression and functional studies *(From Olaf Witt, Cancer Lett. 2009 8 May, 8;277 (1):8-21)*

In acute promyelocytic leukemia (APL) cells (t15;17) and RARα and PLZF (promyelocytic zinc finger) t(11;17), HDAC3 is a key component of the aberrant transcription regulation in PML-RARa-expressing cells. These aberrant proteins bind to RAREs, recruit HDACs with high affinity and are not responsive to retinoids, which results in the constitutive repression of RAR-targeted genes. However, as discussed below, addition of HDAC inhibitors can restore sensitivity of APL cells, indicating that aberrant histone deacetylation is a key process in leukemogenesis[48,49].

Moreover, the targeted inhibition of HDAC6 leads to acetylation of HSP90 and disruption of its chaperone function, resulting in depletion of progrowth and prosurvival client proteins, including the Bcr-Abl onco-protein in K562 leukemic cells[48,50,51].

Collectively, these data show that inappropriate transcriptional repression mediated by HDACs is a common molecular mechanism that is used by onco-proteins, and alterations in chromatin structure can impinge on normal cellular differentiation, which leads to tumor formation.

1.1.3.1 Histone Deacetylation Inhibitor

The anticancer potential of HDAC inhibitors (HDACi) stems from their ability to affect several cellular processes that are dysregulated in neoplastic cells, and represent a new category of anticancer drugs. They constitute therefore exciting prospects for a more rational approach to chemotherapy. It is not yet precisely clear how HDACi exert these effects, however, at the structural level, HDACi have been proposed to prevent histone deacetylation activity

by binding in the active site pocket and chelating the Zn2+ ion of class I and class II Zn2+-dependent HDACs[52-54].

The molecular and biological functions of these agents will be now discussed, and their use in clinical trials will be outlined.

At the molecular level, HDACi increase the histone acetylation which, in turn, leads to the activation of the transcription of a few genes whose expression causes the inhibition of tumor growth, Figure 3[20,55].

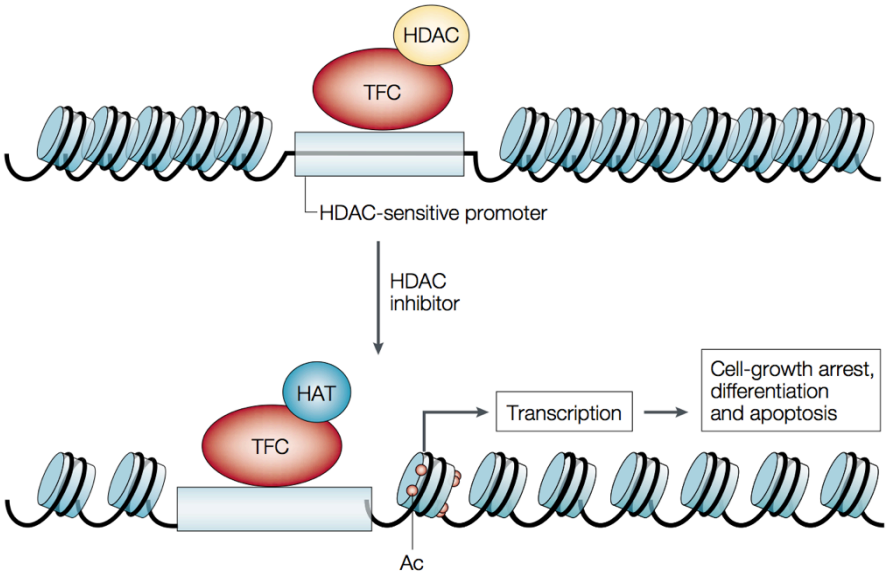

Figure 3. Mechanism of action of histone deacetylase inhibitors. With inhibition of HDACs by HDAC inhibitors such as suberoylanilide hydroxamic acid, histones are acetylated, and the DNA that is tightly wrapped around a deacetylated histone core relaxes. It is propose that there are specific sites in the promoter region of a subset of genes (for example, SP1 sites) that recruit the transcription factor complex (TFC) with HDAC and that the accumulation of acetylated histones in nucleosomes leads to increased transcription of this subset of genes (for example, *CDKN1A*, which encodes WAF1), which, in turn, leads to downstream effects that result in

cell-growth arrest, differentiation and/or apoptotic cell death and, as a consequence, inhibition of tumour growth. Ac, acetyl group. *(Adapted from Marks P.; Nat Rev Cancer; 2001 Dec; 1(3):194-202)*

Increased activity of HDACs leading to reduced or abnormal histone acetylation patterns has been described in cancer cells. It has been shown that malignant cells present with higher level of HDACs than normal tissues, generating hypoacetylated histones. In HDACi treated cells, in addition to the induction of the expression of several genes, the repression of a considarable number of other genes has also been reported. This inhibition may be the consequence of both direct and indirect effects. A gene could be repressed because of the activation of a transcriptional repressor. Alternatively, acetylation of histones could lead to a chromatin conformation that directly recruits a repressor protein rather than a transcriptional activator[56]. Another possibility is that rather than a histone being the substrate for the HDAC, another protein such as a transcription factor could become acetylated. The acetylation could either repress or activate the transcription factor leading to either the repression or activation of transcription, respectively[3,57,58]. The findings that additional proteins are substrates for the HDACs also raise important questions regarding the use of histone acetylation as a surrogate marker of drug activity in clinical trials[4,59]. Perhaps taken together, HDACi elicit their anti-tumor activity through a much broader range of regulatory actions than previously anticipated and highlight the great diversity and complexity by which these drugs function.

1.1.3.2 Histone Deacetylation Inhibitor Classes

A relatively wide range of structures have been identified that work equally well against class 1, class 2 and class 4 HDACs. The common mechanism of action of these drugs is to bind a critical Zn^{2+} ion required for the catalytic function of the HDAC enzyme. They derive from both natural sources and from synthetic routes, and can be categorized into several groupings, in order of decreasing potency[60,61]:

a) Hydroxamic acids (or hydroxamates), such as trichostatin A
b) Cyclic tetrapeptides (such as trapoxin B), and the depsipeptides
c) Benzamides,
d) Electrophilic ketones (epoxides)
e) Aliphatic acid compounds such as phenylbutyrate and valproic acid (VPA).

The chemical structures shown in Fig 4 are representative of compounds from the major classes of HDACi.

The development of HDACIs started with the discovery that several compounds that were known to induce differentiation of leukemic cell lines were inhibitors of HDACs and induced hyperacetylated histones. This led to the development of other compounds with HDACi activity, based on the hypothesis that inhibition of HDACs would induce histone hyperacetylation and restore gene expression in cancer cells. This could potentially lead to cell differentiation, inhibition of proliferation and/or apoptosis.

Many structurally diverse compounds can bind to HDACs

and inhibit their enzymatic activity, including hydroxamates, cyclic peptides, aliphatic acids and benzamides, as described above. Due to the highly conserved nature of the enzymatic pocket, most HDACi do not selectively inhibit individual HDAC isoenzymes and either inhibit all HDACs or at least several members simultaneously[27,62,63].

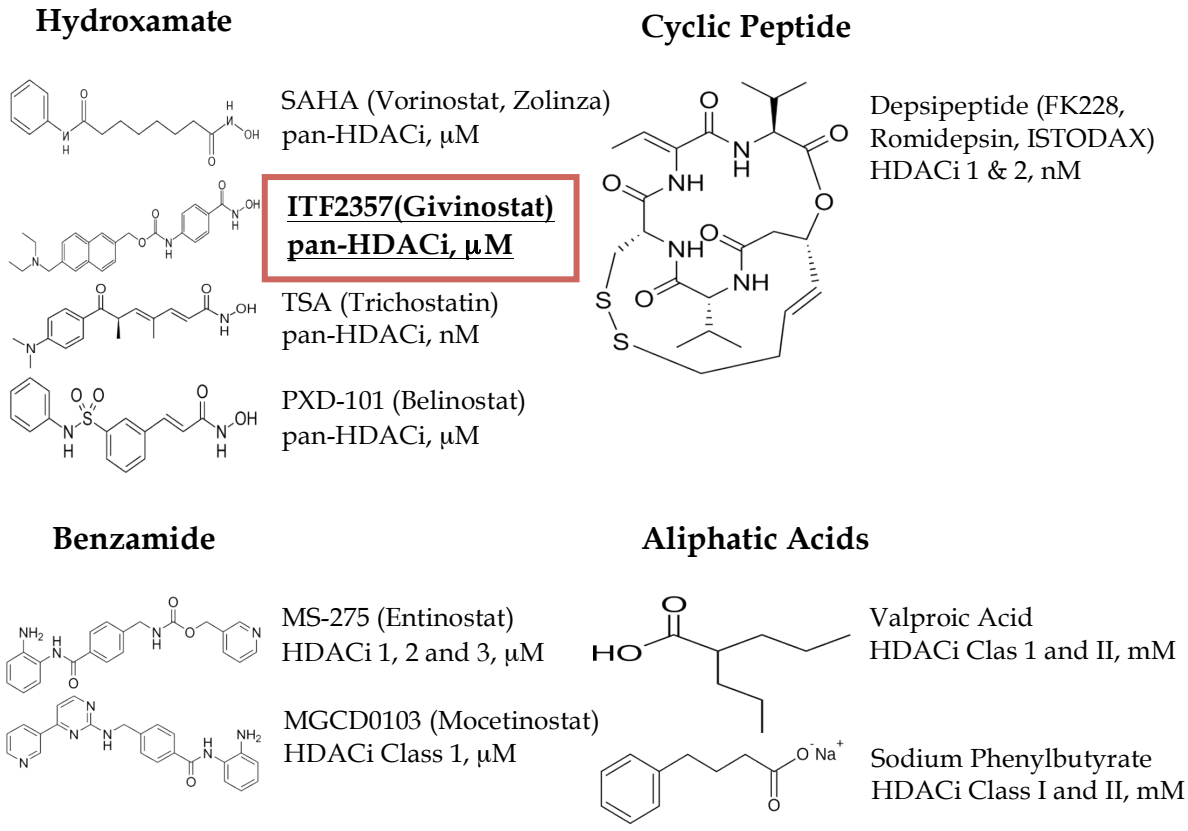

Figure 4. Structures of major classes of HDAC inhibitors. Suberoylanilide hydroxamic acid (SAHA/Vorinostat/Zolinza®), ITF2357 (Givinostat) Trichostatin A (TSA), and PXD-101 are hydroxamic acid-based pan-HDAC inhibitors. Depsipeptide (FK228/ romidepsin/ISTODAX®) is a natural cyclic peptide product of prodrug type, which inhibits HDAC1 and 2 selectively. Both MS-275 and MGCD0103 are synthetic benzamide

derivatives. MS-275 is selective to HDAC 1, 2, and 3, and MGCD0103 is a class I selective HDACi. Aliphatic acids include valproic acid and sodium phenylbutyrate which have relatively low HDACi potency.

Characterization of the inhibitory profile of currently used inhibitors within the entire HDAC family is hampered by the fact that recombinant production of purified, active enzymes is difficult. The situation becomes even more complicated as many HDACs require multi-protein complexes or interaction with other HDACs for full enzymatic activity and therefore, in vitro HDAC activity is unlikely to reflect the true intra-cellular situation.

The first HDACi produced was hexamethylene bisacetamide, and its more potent analogs of the so-called hybrid polar class. A related fungal product, trichostatin A, displayed similar differentiating effects in vitro. The activity of these compounds, all derivatives of hydroxamic acid, prompted the synthesis of vorinostat (suberoylanilide hydroxamic acid-SAHA). They differ in their potency and specificity against different classes of HDACs (Table 5)[64].

Chemical Class	Agent	IR	In vitro effect
Hydroxamates	Vorinostat	μM	Apoptosis, cell-cycle arrest, differentiation
	Panobinostat	nM	Apoptosis, cell-cycle arrest, differentiation
	Givinostat (ITF2357)	μM	Apoptosis, cell-cycle arrest, differentiation
	LAQ824	nM	Apoptosis, cell-cycle arrest, differentiation
	PXD101	nM	Apoptosis, cell-cycle arrest, differentiation
	Trichostatin A	nM	Apoptosis, cell-cycle arrest, differentiation
	Oxamflatin	μM	Apoptosis, cell-cycle arrest
	Scriptaid	μM	Apoptosis, cell-cycle arrest, differentiation
	Pyroxamide	nM	Apoptosis, cell-cycle arrest, differentiation
	CBHA	μM	Apoptosis, cell-cycle arrest, differentiation
Cyclic tetrapeptides	CHAP	nM	Cell-cycle arrest
	Trapoxin	nM	Cell-cycle arrest, differentiation

	Depsipeptide (FK228)	μM	Cell-cycle arrest, apoptosis
	Apicidin	nM	Cell-cycle arrest, apoptosis
Benzamides	Entinostat	μM	Cell-cycle arrest
Epoxide	Depudecin	μM	Cell-cycle arrest, differentiation
Aliphatic acid	Phenylbutyrate	mM	Apoptosis, cell-cycle arrest, differentiation
	VPA	mM	Apoptosis, differentiation
Sulfonamide anilide	MGCD-0103	μM	Apoptosis, cell-cycle arrest

Table 5. Chemical classes of HDACi and biological activities. Abbreviations: IR, Inhibitory Range; CBHA, m-carboxy cinnamic acid bishydroxamic acid; CHAP, cyclic hydroxamic acid-containing peptide (trapoxin analog); SCFA, short-chain fatty acids; VPA, valproic acid. *(Adapted from Quintas-Cardama A.; Leukemia; 2011 Feb; 25(2):226-35)*

Although histone deacetylation has a fundamental role in regulating gene expression, HDACi seem to directly affect transcription of only a relatively small number of genes. Initial studies using differential-display techniques estimate that less than 2% of genes are affected; however, more recent analyses using DNA microarrays put this number closer to 10%[65].

Interestingly a shown in Table 6, diverse HDACi activate a common set of genes, which indicates that certain loci are more susceptible to these compounds than others. Of the genes investigated so far, the effect of HDACi is overwhelmingly geared towards regulating those that control cell growth and survival, providing a mechanistic explanation for the anticancer properties of these drugs[66].

Inhibitor	Activated genes	Repressed genes
Butyrates	*CDKN1A*, *GATA2*, PKCD, *MHC1*, *MHC2*, *BAK*, *IL8*, *RARβ*, *TG1*, cyclin E, *CPA3*, *CD86*, *ICAM1*	Cyclin D1, cyclin A, *BCL2*, *IL2*, $BCLX_L$
Valproic acid	β-catenin	
Trichostatin A	*CDKN1A*, *GATA2*, *HSP86*, *CDKN1B*, *PKCD*, *HDAC1*, *IGFBP3*, *DHFR*, *TGFB1*, *ER*, *CD86*, cyclin E, *IFNG*, *IFNB*, *TP53*, *VHL*, *MHC1*, *MHC2*, *CPA3*, *P107*, *BAX*, *BAK*, *TG1*, *CDNK2A*, *MLH1*, *TIMP3*	Cyclin A, *CDKN1C*, $BCLX_L$, *PU.1*, *HIF1A*, *VEGF*, *IL2*, *IL10*
Suberoylanilide hydroxamic acid (SAHA)	*CDKN1A*	*CMYC*, *CMYB*, *BMYB*
m-Carboxy cinnamic acid bishydroxamic acid (CBHA)	*CD95*, *CD95L*	
Oxamflatin	*CDKN1A*, gelsolin, cyclin E, *CDKN1B*, *PAI2*	Cyclin A, cyclin D
CHAP	*CDKN1A*, *MHC1*	Cyclin A
Trapoxin A	*CDKN1A*, *CD86*, cyclin E	
Apicidin	*CDKN1A*, *CD95*, *CD95L*, gelsolin	
FR901228 (Depsipeptide)	*CDKN1A*, *MAGE3*, *NY–ESO1*, *CD86*	*CD95L*, *CMYC*, cyclin D
Depudecin		
MS-27-275	*CDKN1A*, gelsolin, *TGFBR2*	

Table 6. Most important genes activated or repressed by different HDACi in tumor cells. *BAK*, BCL2 antagonist/killer protein; *BAX*, B-cell-associated X protein; *BCL2*, B-cell lymphoma protein 2; *BCLXL*, BCL2-related protein, long form; CD95L, CD95 ligand; *CDKN1A*, cyclin-dependent-kinase inhibitor 1A; *CDKN1B*, cyclin-dependent-kinase inhibitor 1B; *CDKN1C*, cyclin-dependent-kinase inhibitor 1C; *PCPA3*, carboxypeptidase A3; CT, cyclic tetrapeptide; *DHFR*, dihydrofolate reductase; *ER*, oestrogen receptor; *GATA2*, GATA-binding protein 2; HA, hydroxamic acid; *HDAC*, histone deacetylase; *HIF1A*, hypoxia-inducible factor 1, α-subunit inhibitor; *HSP86*, heat-shock protein (90 kDa); *ICAM1*, intercellular adhesion molecule 1;

IFNB, interferon-β; *IFNG*, interferon-γ; *IGFBP3*, insulin-like-growth-factor binding protein 3; *IL,* interleukin; *MAGE3*, melanoma antigen, family A, 3; MHC, major histocompatibility complex; *MLH1*, mutL homologue 1; *BMYB*, v-*myb* myeloblastosis viral oncogene homolog (avian)-like 2; *CMYB*, v-*myb* myeloblastosis viral oncogene homologue (avian); *CMYC*, v-*myc* myelocytomatosis viral oncogene homologue (avian); *AI2*, plasminogen activator inhibitor type 2; *PKCD*, protein kinase C-δ; PU.1, PU.1 transcription factor; *RAR*β, retinoic-acid receptor-β; SCFA, short-chain fatty acid; *TG1*, transglutaminase type1; *TGFB1*, transforming growth factor-β1; *TGFBR2*, TGF-β receptor 2; *TIMP3*, tissue inhibitor of metalloproteinase 3; *VEGF*, vascular endothelial growth factor; *VHL*, von Hippel–Lindau syndrome. (*Adapted From Ricky W. Johnstone; Nat. Rev. Drug Discov.; 2002 Apr; 1(4):287-99)*

This approach is based on the finding that the expression of a finite number of genes is regulated after exposure to HDACi. Almost all HDACi activate transcription of the $p21^{WAF1}$ (*CDKN1A)* promoter, independent of p53. Furthermore, $p21^{WAF1}$ upregulation was linked to HDACi mediated cell cycle arrest, and only strengthened the model that HDACi achieved their anti-tumor effects as a result of histone hyperacetylation and consequent gene activation[12,67].

1.1.3.3 Antitumour activities of Histone Deacetylation inhibitors

The anticancer potential of HDACi stems from their ability to affect several cellular processes that are dysregulated in neoplastic cells, and almost all them can induce cell-cycle arrest, differentiation or apoptosis *in vitro*, and many have potent anti-tumor activities *in*

vivo[68]. In addition, activation of the host immune response and inhibition of angiogenesis might also have important roles in HDACi mediated tumor regression *in vivo*, as well as their potential direct interaction with non-histone proteins described above, Figure 5[66].

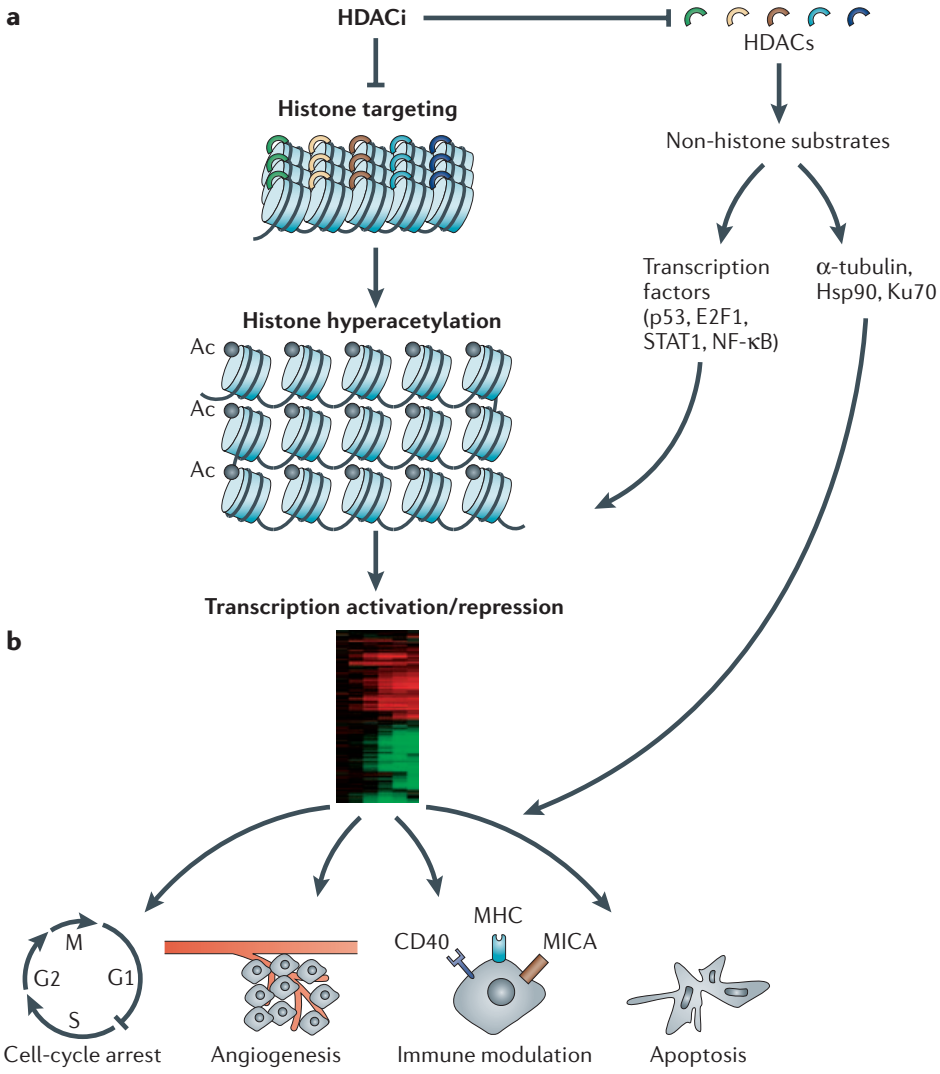

Figure 5. Effects of histone deacetylase inhibition. **a. Effects on non-histone proteins.** In addition to regulating the acetylation state of HDAC can bind to, deacetylate and regulate the activity of a number

of other proteins, including transcription factors (that is, p53, E2F transcription factor 1 [E2F1] and nuclear factor-κB [NF-κB]) and proteins with diverse biological functions (that is, α-tubulin, Ku70 and heat-shock protein 90 [Hsp90]). Hyperacetylation of transcription factors with HDACi can augment their gene-regulatory activities and contribute to the changes in gene expression observed following direct HDACi-mediated histone hyperacetylation. Hyperacetylation of proteins such as Ku70 and Hsp90 or disruption of protein phosphatase 1 (PP1)–HDAC interactions by HDACi might have no direct or indirect effect on gene expression but could be important for certain biological effects of HDACi, in particular the induction of apoptosis and cell- cycle arrest.

b. Antitumor effects of HDACi. HDACi can affect tumor cell growth and survival through multiple biological effects. HDACi induce cell-cycle arrest at the G1/S boundary through upregulation of *CDKN1A* (encoding $p21^{WAF1/CIP1}$) and/or through downregulation of cyclins. HDACi can suppress angiogenesis through decreased expression of pro-angiogenic factors such as vascular endothelial growth factor (VEGF), hypoxia-inducible factor 1α (HIF1α) and chemokine (C-X-C motif) receptor 4 (CXCR4). HDACi can also have immunomodulatory effects by enhancing tumor cell antigenicity (upregulation of major histocompatibility complex class I and II, MICA, CD40) and by altering the expression of key cytokines including tumour-necrosis factor-α, interleukin-1 and interferon-γ. *(Adapted from Jessica E. Bolden; Nat Rev Drug Discov; 2006 Sep; 5(9):769-84)*

Cell-Cycle arrest. HDACi were first discovered as a result of their capacity to induce cellular differentiation and this effect is intimately associated with cell-cycle arrest at the G1/S boundary mediated by the retinoblastoma protein (pRb) and related proteins. All HDACi studied to date, with the possible exception of tubacin, can induce cell-cycle arrest at G1/S (Fig. 5). Almost all HDACi

activate transcription of the cyclin-dependent kinase (CDK) inhibitor WAF1 (also known as CIP1, p21; encoded by the *CDKN1A* locus), which can inhibit cyclin E–CDK2 and cyclin A–CDK2, and many HDACi downregulate cyclins A and D. This results in hypophosphorylation of the retinoblastoma tumor-suppressor protein RB, and inhibition of S-phase progression. Induction of *CDKN1A* is necessary for HDACi-induced G1 arrest and treatment of cells deficient in *CDKN1A* with HDACi results in accumulation of cells with a 4*n* DNA content and sensitization to apoptosis. Moreover two genes are involved in DNA synthesis, *CTP synthase* and *thymidylate synthetase*, and these are transcriptionally repressed by HDACi, therefore the loss of these enzymes would have a similar effect to antimetabolite treatment (eg, Hydroxyurea) that blocks S-phase progression, thereby also contributing to the G1/S arrest. HDACi can mediate G2/M-phase arrest by activating a G2-phase checkpoint, although this is a much rarer event than HDACi-induced G1 arrest and unlike HDACi-mediated G1 arrest, where there is a documented role for the transcriptional activation of *CDKN1A*, the molecular events are unclear[37,38,40,67].

Tumor angiogenesis. Tumor growth requires the development of new vessels that sprout from pre-existing normal vessels in a process known as "angiogenesis". Tumor angiogenesis is often mediated by hypoxia secondary to tumor growth or by increased oncogenic signaling. Both mechanisms result in increased hypoxia-inducible factor-1 alpha (HIF-1α) signaling and its transcriptional target vascular endothelial growth factor (VEGF).

Critical to HIF-1α signaling are post translational modifications including acetylation mediated by HAT and deacetylation by HDAC. HDACi also modulate angiogenesis in a potentially therapeutic manner, the anti-angiogenic properties of them have been associated with decreased expression of pro-angiogenic genes. For example, most HDACi are capable of downregulating VEGF, HIF1α, and others like basic fibroblast growth factor (bFGF), angiopoietin, tunica intima endothelial kinase 2 (TIE2) and endothelial nitric oxide synthase (eNOS), which is commensurate with their inhibitory effects on neo-angiogenesis *in vitro* and *in vivo*.

Recently it has been shown that members of class IIa HDACs in particular HDAC5 is a repressor of angiogenic gene expression in endothelial cells. This possible specific control of angiogenesis may have potential therapeutic implications. Indeed one may consider using HDAC5 inhibitors to improve therapeutic angiogenesis (eg, after ischemia) or to use HDAC5 activators to block pathologic angiogenesis[69,70].

Immunomodulatory effects. HDACi can potentially modulate anti-tumor immunity via numerous mechanisms including upregulation of tumor antigens, enhancement of cellular immune recognition, lysis of tumor targets by T cells and NK cells, and alteration of T-cell subsets as well as inflammatory cytokine profiles. In this context, there is increasing evidence that HDACi can enhance anti-tumour immunity either by directly affecting malignant cells so as to make them more attractive immune targets, or by altering immune cell activity and/or cytokine production. HDACi can augment the immunogenicity of tumor cells by upregulating the

expression of major histocompatibility complex (MHC) class I and II proteins, and co-stimulatory/adhesion molecules such as CD40, CD80, CD86 and intercellular adhesion molecule 1 (ICAM1). The gene-regulatory activities of STAT1 (signal transducer and activator of transcription 1), STAT3 and nuclear factor-κB (NF-κB), which are often considered to be 'master immune regulatory transcription factors', are directly regulated by acetylation, which raises the intriguing possibility that changes in cytokine profiles and/or effects on immune cell function that occur following HDACi treatment might be a result of altered acetylation of these factors[71] .

Several recent studies indicate that HDACi can modulate TH1/TH2 effector function, and enhance the activity of FOXp3-positive regulatory T cells, which contribute to immune tolerance in cancer patients. Furthermore, TSA abrogates interferon-gamma (IFN-γ)-mediated inhibition of TNF-α- induced activation of inflammatory cytokine genes such as IL-6 and IL-8, which enhance metastatic potential of cancer cells. The exact role of HDACi-mediated immunomodulation in the observed cancer response remains to be determined in either preclinical or clinical settings[8].

HDACi-mediated apoptosis. There are preclinical evidence of pleiotropic cytotoxic effects of HDACi of diverse structural classes in cultured cancer cells and various human tumor xenografts and treatment of various tumor cells with HDACi can induce tumor cell death with all of the biochemical and morphological characteristics of apoptosis. The therapeutic potential of HDACi stems from their capacity to selectively induce apoptosis in tumor cells, including breast, prostate, lung and thyroid carcinoma, leukemia, and multiple

myeloma[66]. Evidence for tumor-selectivity comes from the preclinical animal experiments and clinical trials demonstrating that HDACi can have potent anti-cancer activities at concentrations that are minimally toxic to the host, sine normal cells are almost always considerably more resistant than tumor cells to HDACi. Dose-limiting toxicities include thrombocytopenia, nausea and fatigue; in the case of depsipeptide, cardiac arrhythmia was additionally observed[72]. In many cases these adverse effects are clinically manageable; however, it was perhaps overly optimistic to believe that treatment with HDACi would not result in any unfavourable side effects.

HDACi induce, to a variable extent, growth arrest, differentiation or apoptosis *in vitro* and *in vivo*[73]. In some cases, growth arrest is induced at low doses, and apoptosis is induced at higher doses; in other cases, growth arrest precedes apoptosis[74].

In vitro studies showing that transformed cells may be at least tenfold more sensitive to HDACi compared with normal cells provide some direct evidence for HDACi- mediated, tumor-cell-selective killing. Although it is irrefutable that HDACi can kill tumor cells, the molecular pathways that are engaged to mediate this effect remain to be fully elucidated. In addition exposure times for *in vitro* apoptosis assays can differ markedly from those achievable *in vivo*, and there for it has not yet been conclusively demonstrated that the therapeutic activities of the compounds are directly related to their capacity to induce tumor cell death. Given the pleiotropic biological effects of HDACi, it is unlikely that a single molecular pathway leading to tumor cell death will be identified for all HDACi in all cell types.

In order to explain the mechanisms of apoptosis induced by HDACi, it is necessary to know that trigger apoptosis. Apoptosis proceeds through one of two functionally separable but molecularly linked intracellular death pathways that require a family of cysteine proteases (caspases) see Figure 6.

One (extrinsic) death-receptor pathway, is activated when ligands, such as Fas or TRAIL, bind to their death receptors. After ligation, the activation of membrane-proximal caspases such as caspase-8 and -10, takes place which in turn cleave and activate effector caspases such as caspase-3 and -7[75,76].

The second (intrinsic) mitochondrial pathway is activated by stress stimuli (chemotherapeutic agents) that disrupt the mitochondrial membrane, causing the release of proteins, including cytochrome *c* and SMAC. Cytochrome *c* release leads to apoptosome formation and activation of caspase-9. Caspase-8 and caspase-9 can then cleave caspases- 3, -6, and -7, culminating in apoptosis[77].

Various pro-apoptotic (BCL2-associated X protein (BAX) and BCL2 antagonist/killer protein (BAK) and anti-apoptotic (B-cell lymphoma 2 (BCL2) and BCL-XL) members of the BCL2 family of proteins regulate these cell death pathways[65].

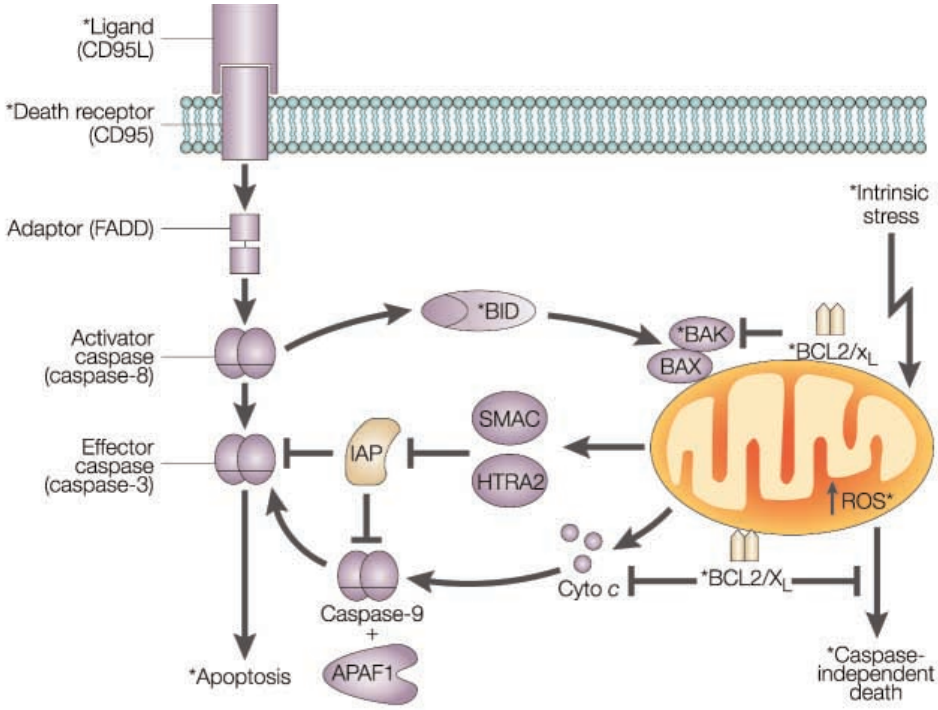

Figure 6. Induction of cell death by HDACi. HDACi can activate both the death-receptor and intrinsic apoptotic pathways. The death-receptor pathway is triggered by the ligation of death receptors, which results in binding of adaptor proteins (FADD) and the recruitment and activation of membrane-proximal activator caspases (caspase-8). These caspases in turn activate downstream effector caspases (caspase-3). The intrinsic apoptotic pathway is activated by internal stresses that induce mitochondrial membrane disruption mediated by pro-apoptotic BCL2 proteins (BAX/BAK). HDACi can induce the activation of the intrinsic apoptotic pathway, and anti-apoptotic BCL2 proteins inhibit mitochondrial membrane damage. HDACi might still induce cell death in the absence of caspase activation after the production of reactive oxygen species (ROS). Asterix's denote components of the death pathways that are affected by HDACi. Those components that inhibit cell death are shown in yellow, and those that promote death are in purple. BAK, BCL2 antagonist/killer protein; BAX, B-cell-associated X protein; BCL2, B-cell lymphoma 2; Cyto *c*,

cytochrome *c*; FADD, FAS-associated via death domain; HTRA2, high temperature requirement 2. (*From Ricky W. Johnstone; Nat. Rev. Drug Discov.; 2002 Apr; 1(4):287-99)*

All HDACi have been reported to activate either the extrinsic (death receptor) or intrinsic (mitochondrial) pathway or both, depending on the cell type and/or the HDACi under investigation:

Death-receptor (extrinsic) pathway. Activation of the extrinsic pathway by HDACi occurs through transcriptional up-regulation of various TNF receptor super-family members and/or their cognate ligands. Indeed, studies by different groups using various genetic or biological means to inhibit death receptor signaling have demonstrated that death receptor signaling is required for HDACi-induced apoptosis. It was observed that MS275 (entinostat) as well as SAHA induced TRAIL expression without altering DR4 or DR5 levels in breast cancer cells, which was mediated via SP1 and markedly enhanced adriamycin cytotoxicity in these cells. This resulted in the recruitment of an adaptor protein, FADD, and the activation of caspase-8.

Mitochondrial (intrinsic) death pathway. A large number of independent studies strongly support a role for the mitochondrial apoptotic pathway in HDACi- mediated tumour cell death. In fact overexpression of *BCL2*, which can block the intrinsic cell-death pathway while leaving the death-receptor pathway intact, inhibits HDACi-mediated apoptosis, indicating the importance of the intrinsic pathway for the function of HDACi. SAHA and MS275 induced mitochondrial dysfunction and apoptosis through enhanced ROS generation, XIAP downregulation and JNK1 activation.

Death-receptor and Mitochondrial pathway. HDACi, including TSA, FK228, SAHA, and LBH589, decrease the expression of Bcl-2, Bcl-xL and XIAP, and enhance the expression of pro-apoptotic proteins, such as Bax and Bak, thereby enhancing TRAIL-mediated cytotoxicity in a variety of cancer cells via the amplification of intrinsic as well as extrinsic apoptotic pathways

Effects of HDACi on non-histone proteins. Although the anti-cancer effects of HDACi detailed in the above sections have mainly been linked to the capacity of HDACi to directly regulate gene expression through histone hyperacetylation, it is clear that histones are not the only molecular targets of HDACs and HDACi can therefore affect tumor cell biology in ways that do not directly involve histones. For example, the gene regulatory activity of transcription factors such as E2F1, p53, STAT1, STAT3 and NF-κB can be modulated through direct acetylation and deacetylation of the factors themselves, and all of these proteins are hyperacetylated in response to HDACi. Consequently, the expression of downstream target genes of these factors can be affected following HDACi treatment not through direct promoter hyperacetylation of these genes, but through the activity of hyperacetylated transcription factors[66,78].

Furthermore, an additional mechanism of apoptosis may be reconducted to HDACi interaction with Heat-shock protein 90 (Hsp 90). Indeed Hsp90, is an abundant cellular chaperone whose overexpression in tumor cells correlates with poor prognosis and resistance to chemotherapy. As part of a multi-protein complex that includes Hsp70, Hsp90 binds a diverse array of client proteins, including key oncogenic and anti-apoptotic proteins, and prevents

their ubiquitinylation and proteasomal degradation. Hsp90 is deacetylated by HDAC6 and HDACi capable of inhibiting HDAC6 induce the hyperacetylation of Hsp90, which leads to the proteasomal degradation of Hsp90 client proteins HER2/*neu*, ERBB1, ERBB2, Akt, c-Raf, BCR–ABL and FLT3[79,80].

In conclusion, many areas of investigation on the biological effects of HDACi show an exciting and complex network of cellular responses, coupling transcriptional and non-transcriptional effects. It is difficult at this stage to predict the existence of a universal, key cellular target that is responsible for the HDACi-mediated response. An encouraging observation is that in several cases, normal cells show a strikingly reduced sensitivity to HDACi treatment, hinting at potentially large differences in the acetylome in normal versus tumour cells that can be exploited clinically.

1.2 Description of Givinostat (ITF2357)

The HDACi Givinostat (ITF2357) is a hydroxamate pan-HDACi (Italfarmaco, S.p.A.), with potential anti-inflammatory, anti-angiogenic, and antineoplastic activities. ITF2357 is an HDACi that contains a hydroxamic acid moiety linked to an aromatic ring, as described in patent WO 97/43251, US 6034096 and shown in Figure 4. The compound was synthesized at Italfarmaco (Cinisello Balsamo, Italy), and first data were published in 2005[81]. Currently Givinostat (GVS) has orphan drug designation for the treatment of polycythaemia vera[82,83].

1.2.1 In vitro activity of GVS against hematologic tumors

HDACi have anti-tumoral proprieties able to induce cell death and cell cycle arrest in tumor cells. The anti-tumor properties GVS were therefore investigated. GVS was studied against multiple myeloma (MM)[83], acute myeloid leukemia (AML) and acute lymphoid leukemia (ALL) cell lines and primary cells freshly isolated from patients. Several parameters were evaluated[84-86]. They include cytotoxicity, apoptosis, cell cycle and anti-clonogenic potential. Furthermore, since the growth of MM cells is largely dependent on their interaction with bone marrow stromal cells and the growth factors that these cells produce, the effect of GVS on the production of IL-6 by stromal cells and on the growth of IL-6 dependent cell line was studied. SAHA, a HDACi structurally related to GVS and recently approved for CTCL treatment, was used as a comparator in many of these studies[87,88].

Cytotoxicity and anti-proliferative effect. Cell viability, measured after 24 or 48 hours incubation, was reduced in most cell lines by concentrations of GVS ranging from 0.06 to 1 μM (median range 0.1 – 0.3 μM). For cell viability reduction in the GFD8 (AML) and REH (ALL) lines longer incubation times were necessary. SAHA was also cytotoxic although less so than GVS. The following table 7 summarizes the results obtained:

	CELL LINE	IC_{50} (μM)	
		ITF2357	SAHA
MM	*RPMI 8266*	*0.2*	*0.7*
	H929	*0.2*	*0.7*
	JJN3	*0.2*	*>1.0*
	U266	*1.0*	*>1.0*
	IM9	*0.9*	*>1.0*
	KMS 11	*0.3*	*1.0*
	KMS 12	*0.2*	*0.9*
	KMS 18	*0.1*	*0.9*
	KMS 20	*0.1*	*0.8*
(ALL)	CEM (T-ALL)	0.07	0.2
	RS4.11 (Pre-B)	0.09	0.2
	TOM1 (pre-B)	0.35	> 1
	697 (cALL)	0.06	0.25
	REH (pre-B)	> 1	> 1
(AML)	HL-60 (M2)	0.26	0.80
	THP1 (M5)	0.28	0.97
	U937 (M5)	0.40	>1
	KASUMI (M2)	0.08	0.27
	KG-1 (M1)	0.07	0.91
	TF-1 (M6)	0.26	>1
	GFD8 (M1)	>1.0	>1

Table 7. Response of MM,ALL and AML cell lines to GVS (ITF2357) and SAHA

Anti-clonogenic potential. The MM cell lines, RPMI 8226 and KMS 11 and the AML cell line KG1 were used to study the ability of GVS and SAHA to inhibit the formation of colonies. The drugs were added to the medium at the beginning of the assay and the number of colonies measured after 21 (MM) or 16 (AML) days. GVS abrogated the formation of colonies in both MM cell lines. The IC_{50} was about 125 nM. Similarly the IC_{50} value in the KG-1 assay was 80nM. In general SAHA was less efficacious than GVS. The following figure 7 summarizes the results obtained:

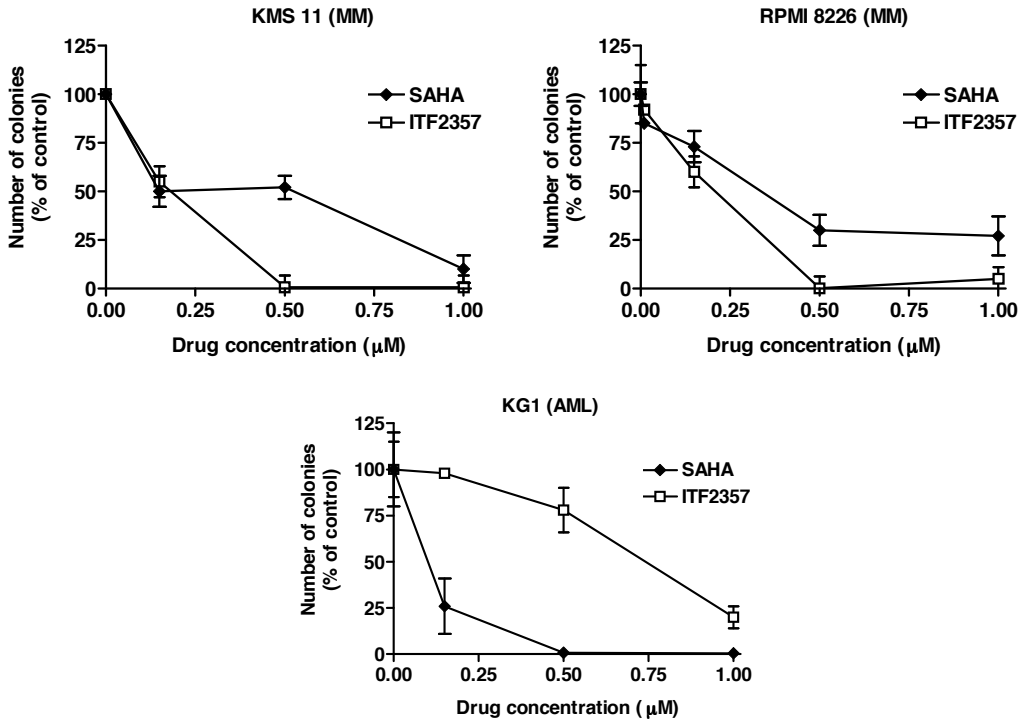

Figure 7. The effect of GVS (ITF2357) and SAHA on colony formation by MM and AML cell lines.

In view of therapeutic application, it is important to know the minimum time of exposure to drug which determines a significant reduction of stem cells colony formation. To this aim CD34-positive stem cells from healthy donors were treated with serial doses of GVS or SAHA for increasing periods (7, 24 and 30 hours). After drug removal, culture was continued and at day 21 the results compared with those from cells treated continuously for 21 days. The results are summarized in the following figure 8:

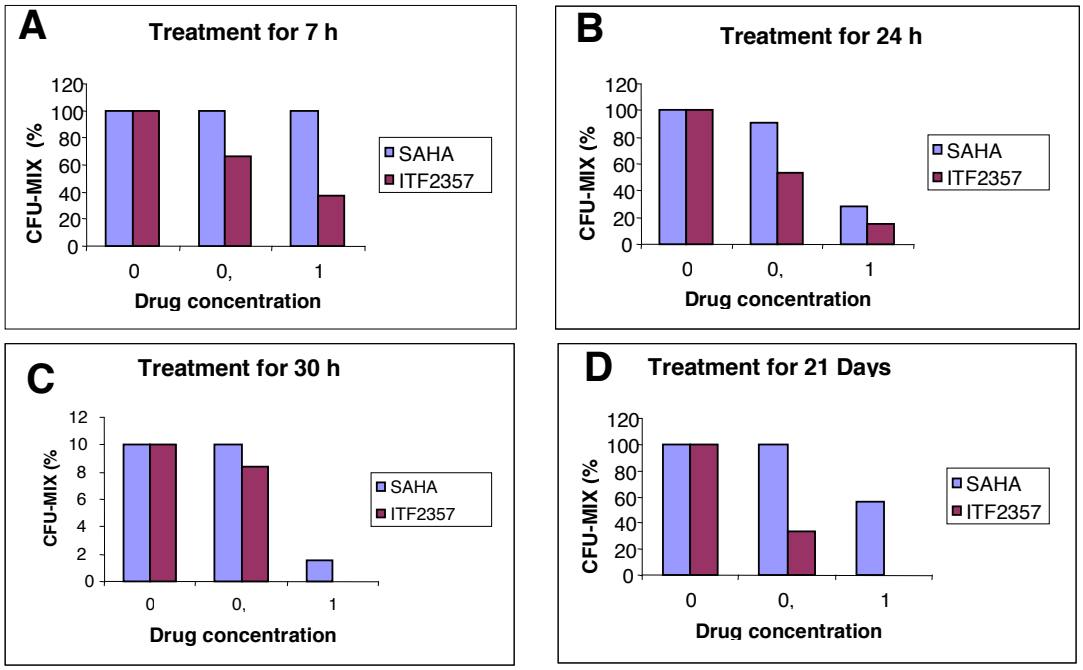

Figure 8. Treatment of primary hematopoietic stem cells with GVS (ITF2357) or SAHA for different times.

Seven hours of treatment with GVS were sufficient to obtain a significant reduction in colonies of CD34-positive stem cells. About 60 and 30% inhibition occurred at 1000 and 100 nM, respectively (Panel A). In contrast, SAHA did not inhibit colony formation at either dose. After 24 hours of incubation (Panel B) the inhibitory effect of GVS increased at both doses whereas SAHA showed a significant effect at 1000 nM only (about 70% inhibition). Prolonging the treatment to 30 hours (Panel C) did not induce further improvement. Thus 7 hours of treatment are sufficient for GVS to exert a biologically relevant inhibitory effect on colony formation. Conversely, SAHA required larger doses and longer exposure times to exert an inhibitory effect on colony formation[83].

Apoptosis. Upon incubation with GVS for 48 hours a dose-dependent increase in apoptosis was seen in MM or AML cell lines as measured by the expression of Annexin V or the expression of activated Caspase-3. The number of necrotic cells also increased upon treatment with GVS.

A similar pattern of cell death (necrosis and apoptosis) induction was observed with SAHA, although 4.5 fold larger doses were required to achieve the same extent of cytotoxicity compared to GVS.

Cell-cycle arrest. The effect of GVS on cell cycle was studied by flow cytometry analysis in RPMI8266 (MM) and M-07e, a megakaryoblastic human cell line.

As demonstrated for other HDACi, GVS induced G1 and/or G2 arrest depending on the cell line. For example at 100 nM a slight decrease in S phase and a corresponding increase in G0/G1 phase were seen in M-07e after 16 hours incubation. After 24 hours at 300 and 1000 nM the apoptotic population was about 20 and 50% of cell population, respectively and the G0/G1 phase increased with concomitant decrease in G2/M and S phases. After 48 hours incubation, the cell cycle progression was heavily modified at all concentrations; no G2/M phase was seen at 300 nM and 100% of cells were in apoptosis at 1000 nM. Similar results were obtained on the human MM cell line RPMI8266.

Cell differentiation. Treatment with HDACi alone or in combination with all-*trans* retinoic acid (ATRA) has been shown to overcome the inhibition of leukemia cell differentiation due to chimeric fusion oncoproteins such as PML-RARα (t(15;17)) in APL and AML-ETO (t(8;21)) in AML, by reverting the HDAC-mediated

repression exerted by these fusion proteins. In addition, a combined treatment with HDACi and ATRA induces differentiation of APL and AML cells that are resistant to ATRA alone. Vitamin D3 is also known to differentiate myeloid leukemia cell lines[89].

1.2.2 Toxicology studies on GVS:

Acute toxicity. In acute toxicity studies in mice and in rats, the median lethal dose of GVS was respectively greater than 150 mg/kg and 132 mg/kg by the intravenous route and greater than 1000 mg/kg and 2400 mg/kg by the oral route. In acute toxicity studies in rodents, dogs and monkeys, the main adverse effects observed were reduction in white blood cells, reduced weight of liver and thymus, bone marrow atrophy and liver and kidney function impairment.

Chronic toxicity. In multiple dose toxicity studies in rats, dogs and monkeys, the main adverse effects observed were bone marrow atrophy, reduced body weight, alterations in hematology and blood chemistry, slight hepatotoxicity characterized by transaminase and bilirubin elevations, and increased triglycerides, urea and creatinine. In the chronic toxicity studies, the no-adverse effect level was established at 10 mg/kg/day.

1.3 Classic Myeloproliferative Neoplasm

1.3.1 Normal hematopoiesis

Hematopoiesis is a delicately regulated process that results in the production of a variety of cell lineages with diverse function that range from delivering oxygen to all tissues to immunity, and hemostasis. This highly regulated process requieres the division of hematopoietic stem cell that give rise to committed progenitors which proliferate and differentiate into functional blood cells. Originated from a small number of multipotential progenitor stem cells in the bone marrow, there are two basic lineages of the hematopoietic system: myeloid and lymphoid stem cells. The stem cells can further differentiate into all mature hematopoietic cell types thereby stepwise loosing their multipotent abilities. Lymphoid stem cells further differentiate into B and T cells whereas megakaryocytes, granulocytes, monocytes and erythrocytes are originate from the myeloid stem cells[90].

The passage of hematopoietic cell through different stages (proliferation, differentiation and maturation of stem and progenitor cells) requieres the presence of multiple hematopoietic growth factors, and these influence transcription factors and implicit associated genes that determine the differentiation and function of mature blood cells (Figure 9)[91]. Perhaps hematopoiesis is controlled by the combined effects of growth factors that permit cellular proliferation and nuclear reagulator (transcription factors) that activate lineage-specific genes[92]. Critical regulators have been

identified both through the study of nuclear factors binding cis-regulatory elements involved in lineage-specific gene expression and by pursuit of genes aberrantly activated in leukemia.

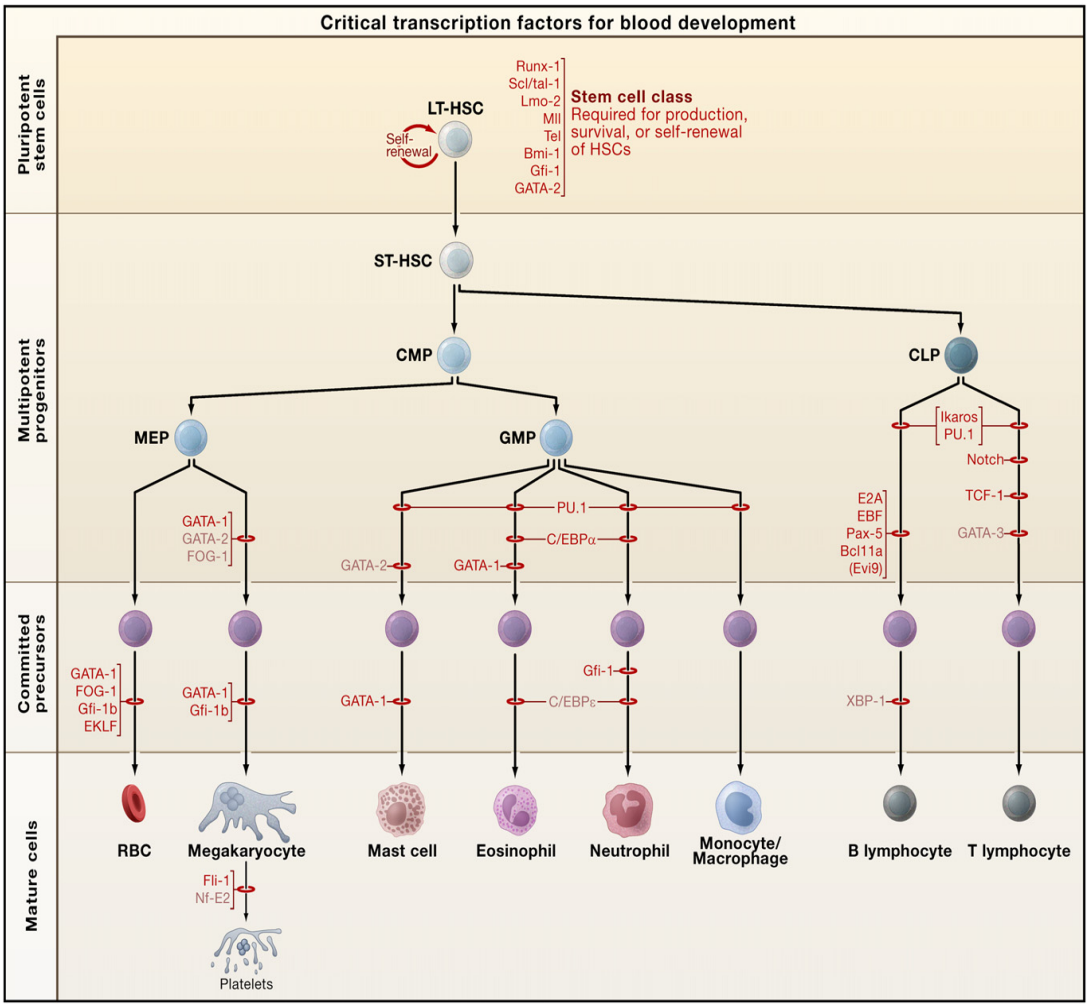

Figure 9. Critical transcription factor for blood development. The stages at which hematopoietic development is blocked in the absence of a given transcription factor, as determined through conventional gene knockouts,

are indicated by red bars. The factors depicted in black have been associated with oncogenesis. Those factors in light font have not yet been found translocated or mutated in human/mouse hematologic malignancies. Abbreviations: LT-HSC, long-term hematopoietic stem cell; ST-HSC, short-term hematopoietic stem cell; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte/erythroid progenitor; GMP, granulocyte/macrophage progenitor; RBCs, red blood cells. (*From Orkin SH; Cell; 2008 Feb 22; 132(4):631-44)*

There are many neoplastic disorders in the hematopoietic system termed "leukemia". In acute leukemia, immature and undifferentiated cells hyperproliferate in the bone marrow, and causes large numbers of abnormal blood cells to be produced and enter the bloodstream, the blood cells are very abnormal, and cannot carry out their normal work, and the number of abnormal cells increases rapidly. In chronic leukemia, the gradual proliferating cells appear mature. With respect to the acute leukemia, the hematopoietic disorders develop much more slowly. Depending on the subtype of the proliferating cell, the clinical course is much more indolent.

Cytokines may have stimulatory, costimulatory/augmenting, inhibitory, or multiple different activities on hematopoietic progenitor/stem cells. To mention briefly we can say that the colony-stimulating factors (CFSs) can induce hematopoietic colony formation in vitro even when used as single factors in the abscence os serum. Most of these factors when used alone exert preferential growth-promoting activity on a single blood lineage[93]. The CSFs includes granulocyte-macrophage colony-stimulating factor (GM-CSF) that induce proliferation and formation of granulocyte-macrophage and eosinophil colonies when added alone to hematopoietic cell cultures.

The granulocyte colony-stimulating factor (G-CSF) induce the proliferation of granulocyte progenitors and their differentiation into granulocytes. The Macrophage colony- stimulating factor (M-CSF), also knowk as CSF-1, stimulates macrophage colony growth from hematopoietic progenitors[94].

Erythropoietin (EPO) is a hormone in the sense that it is produced by the kidney and to a lesser level by the liver, and it is released to the circulation. Promotes the proliferation and maturation of erythroid cell lineages interacting with the receptors on erythroid burst-forming units (B-FUE) and erythroid colony- forming units (E-CFU)[95].

Thrombopoietin (TPO), is produced in the liver, kidney, smooth muscle, and bone marrow among other issues. Also known as c-Mpl ligand, is the primary physiological growth factor for the megakaryocyte (MK) lineage, which also plays a central role in the survival and proliferation of HSC. TPO is the most potent cytokine for stimulating the proliferation and maturation of MK progenitor cells. It stimulates MKs to increase in cell size and ploidy, and to form proplatelet processes that then fragment into single platelets[23,96].

Interleukins (IL) are produced by different types of cells, these participate in the development and differentiation of hematopoietic cells and regulate the immune system.

1.2.2 Myeloproliferative neoplasms

Myeloproliferative neoplasms (MPN) are a heterogenous group of clonal hematological malignancies that are characterized by the expansion of one or more myeloid cell lineages (erythroid, granulocytic, megakaryocytic, monocyte/macrophage, or mast cell) and the main clinical features are the overproduction of mature, functional blood cell and chronic course with a predisposition to leukemia transformation[97] (for the different subtypes of MPN see below).

In 1951, William Dameshek, was the first to classificate these distinct entities. He recognized that many polycythemia vera (PV) patients, not only show the characteristic erythrocytosis, but also have a proliferation of megakarycytes and granulocytes, and it was then he coined the term "myeloproliferative disorders" to embrace these related conditions.

The MPN is currently recognizes on 8 types by 2008 World Health Organization system: chronic myelogenous leukemia, chronic neutrophilic leukemia, polycythemia vera, primary myelofibrosis, essential thrombocythemia, chronic eosinophilic leukemia, mastocytosis, and myeloproliferative neoplasm, unclassifiable. All these can be further categorized by the presence of the philadelphia chromosome Table 8[103,104].

Table 8. MPN Recognized by the World Health Organization Classification of Tumors of Hematopoietic and Lymphoid Tissue

MPN	
Philadelphia Chromosome "positive"	**Philadelphia Chromosome "negative"**
Chronic myelogenous leukemia (CML)	Polycythemia vera (PV)
	Essential thrombocytosis (ET)
	Myelofibrosis (MF)
	Chronic neutrophilic leukemia
	Chronic eosinophilic leukemia
	Mastocytosis
	MPN unclassifiable

The three main Philadelphia negative MPN: PV, ET, and PMF are at the center of our interest. For so, the cardinal features of the three main myeloproliferative disorders are an overproduction of red-cell mass in PV, a high platelet count in ET, and bone marrow fibrosis in PMF (Figure 10). These three disorders share many characteristics, including marrow hypercellularity, a propensity to thrombosis and hemorrhage, and a risk of leukemic transformation in the long term. Monoclonality is the hallmark of these disorder and defines their malignant nature, and these is demostrated by the in vitro formation of endogenous erythroid colonies (EEC)[105] or endogenous megakaryocytic colonies (EMC) in the abscence of added cytokines/growth factors, and using standardized media has been demonstrated, and has led clinicians to consider EEC a minor diagnostic criterion in the WHO 2008 classification[97,103].

The annual incidences of both PV and ET are 1 to 3 cases per 100,000 population; PMF is less common.

In each disorder, all three cell lineages (myeloid, erythroid and megakaryocytic) are involved and the predominant cell lines

allow a subclassification of the disorders. For example, in PV patients the proliferation is predominately erythroid, but granulocytes and megakaryocytes are also part of the malignant clone.

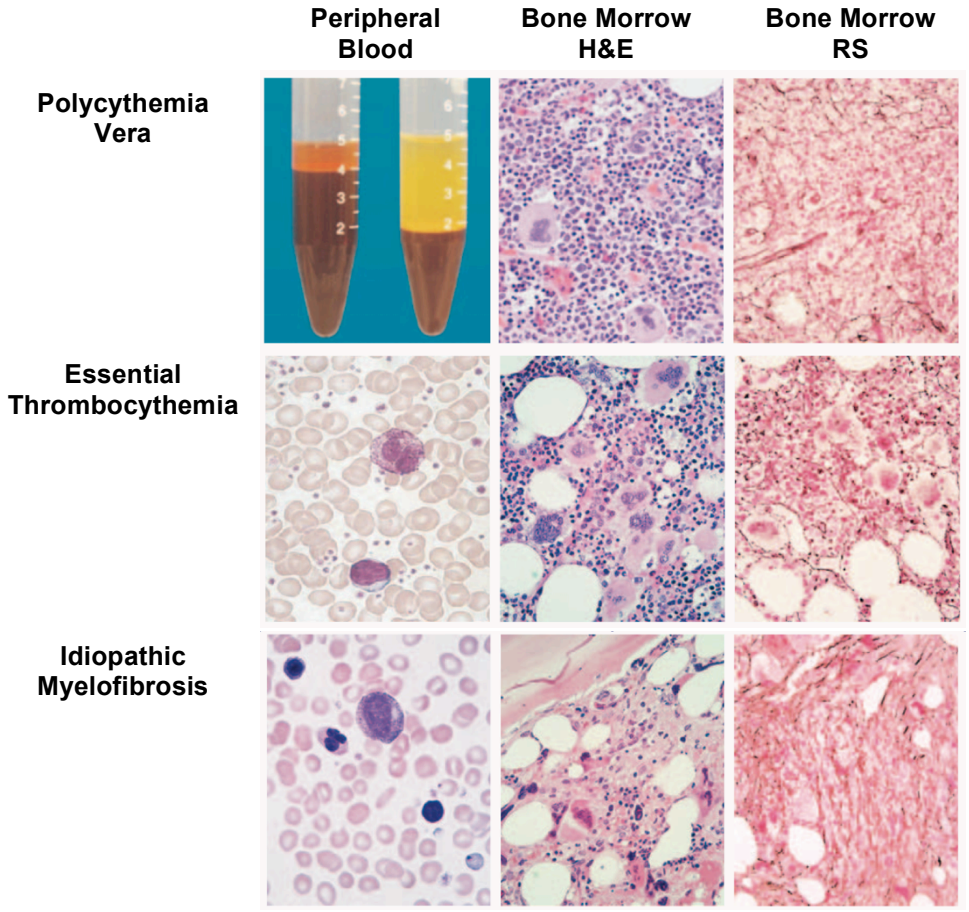

Figure 10. Laboratory Features of PV, ET, and PMF. PV is characterized by an increased hematocrit in the peripheral blood (test tube on left); a hypercellular marrow with increased numbers of erythroid, megakaryocytic, and granulocytic precursor cells; and a variable increase in the number of reticulin fibers. ET is characterized by an increase in the

number of platelets in the peripheral blood and an increased number of megakaryocytes in the marrow, which tend to cluster together and have hyperlobated nuclei. PMF is characterized by the presence of immature red and white cells (a so-called leukoerythroblastic blood film) and "teardrop" red cells, disordered cellular architecture, dysplastic megakaryocytes, new bone formation in the marrow, and the formation of collagen fibers. (*From Campbell PJ.; N Engl J Med; 2006 Dec 7; 355(23):2452-66)*

The MPN not only are phenotypically similar but can also evolve into each other. From a clinical perspective, as illustrated in Figure 11[106] isolated thrombocytosis of unknown cause should not automatically be assumed to be essential thrombocytosis because isolated thrombocytosis can also be the presenting manifestation of PV or PMF.

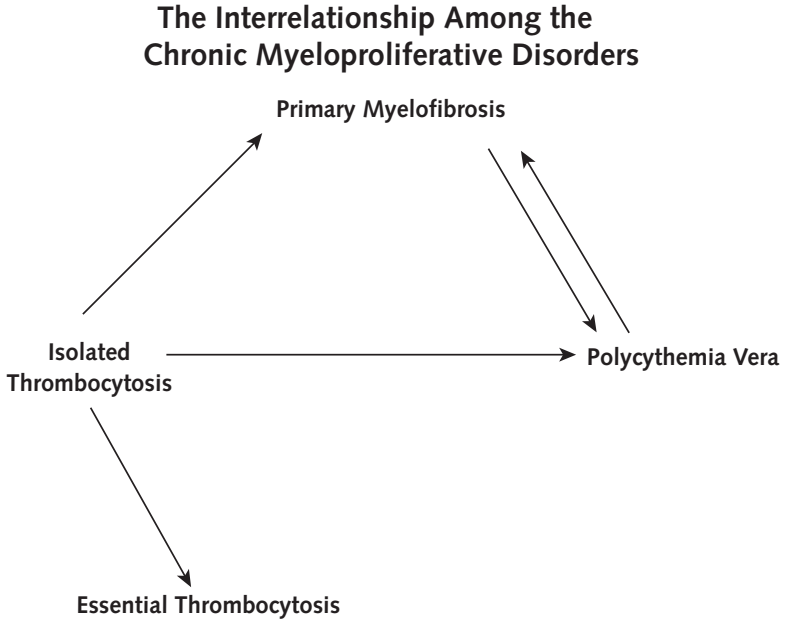

Figure 11. The close interrelationship among the MPNs. (*From Spivak JL.;*

Nonetheless, MPN patients are at a variable predisposition to risk of transformation to AML, which is hypothesized to occur by acquisition of additional genomic abnormalities and is generally associated with a poor prognosis. The incidence of chronic-phase MPN is two to five cases per 100,000 population per year, whereas leukaemic evolution occurs in 2–5% of these patients with either PV or ET and roughly 20% of cases with PMF[107]. High-resolution single-nucleotide polymorphism (SNP) array shows that after leukemic transformation, there are up to 3-fold more genomic changes than in chronic phase[108].

In 1960 chronic myeloid leukemia (CML) (it will not be argued in detail here) became the first cancer to be defined by molecular marker, the Philadelphia chromosome, t(9;22)(q34;q11.2), that led in 1985, to the identification of the consequences of the Philadelphia translocation (the *BCR-ABL* fusion gene), this encodes a chimeric protein that has dysregulated tyrosine kinase function. This fundamental observation ultimately led to the identification of the disease-causing mutation, establishment of accurate diagnostic tests, and development of molecularly targeted therapy.

1.2.3 Polycytemia Vera

In 1892, PV was first phenotypically described by Louis Henri Vaquez and William Osler in patients with elevated erythrocytosis and hepatosplenomegaly. The clonal stem cell population produces excessive numbers of erythrocytes, usually accompanied by

increased platelets and granulocytes as well. The average age is about 60 at diagnosis, which, in asymptomatic patients, may be suspected by finding plethora and splenomegaly on examination or abnormalities on a routine blood count. Symptoms typically arise from the erythrocytosis, which causes hyperviscosity and a tendency for venous and arterial thromboses, such as myocardial infarctions, strokes, and venous thromboses of the legs[98,105].

Early studies in untreated PV patients found a high incidence of thrombotic events and a life expectancy of about 18 months after diagnosis. Cytoreductive treatments of blood hyperviscosity by phlebotomy or chemotherapy have dramatically reduced the number of thrombotic events, even though hematologic transformations toward PMF and acute leukemia still represent a major cause of death[109].

In the 2008 WHO Diagnostic Criteria for PV are in Table 9:

	PV
Major criteria	1. Hemoglobin values should be >18.5g/dL (men) and >16.5g/dL (women) or elevated red cell mass >25% above normal value. 2. Presence of *JAK2*V617F mutation or other similar mutation.

Minor criteria	1. Hypercellular bone marrow with prominent erythroid, granulocytic and megakaryocytic proliferation. 2. Serum erythropoietin below normal levels. 3. In vitro erythroid colony formation.

Table 9: (*From Tefferi A, Leukemia. 2008 Jan; 22(1):14-22)*

The diagnosis of PV requires meeting either both major criteria and 1 minor criterion or the first major criterion and 2 minor criteria.

1.2.4 Essential Thrombocythemia

Of the classic MPNs, ET was last described by Emil Epstein and Alfred Goedel in patients with thrombocytosis with marked erythrocytosis in 1934. In ET a clonal population of mature megakaryocytes in the bone marrow produces excessive numbers of platelets. The average age at diagnosis is about 50 to 60 years, and most cases are discovered by routine blood tests of asymptomatic patients, considering a diagnosis of ET when there is an unexplained and persistent thrombocytosis (platelet count > 450 X 10^9/L). ET has traditionally been a diagnosis of exclusion, requiring the absence of reactive conditions and other clonal disorders that may present with thrombocytosis[89,109,110].

When symptoms occur, they usually are related to vessel thrombosis or abnormal vascular reactivity, such as dizziness,

headaches, visual disturbances, transient ischemic attacks, digital ischemia, and paresthesias[110].

In the 2008 WHO Diagnostic Criteria for ET are in Table 10[103]:

ET
1. Platelet count ≥450x10^9/L.
2. Bone marrow biopsy showing megakaryocyte proliferation with large and mature morphology.
3. Not meeting WHO diagnostic criteria for PV, PMF, CML, MDS or other MPN.
4. Presents of *JAK2*V617F mutation or other clonal marker, or no evidence of reactive thrombocytosis in the absence of *JAK2*V167F.

Table 10: (*From Tefferi A, Leukemia. 2008 Jan; 22(1):14-22)*

All four criteria must be met when make diagnosis.

1.2.5 Primary Myelofibrosis

Myelofibrosis was frist described by Gustav Hueck in 1879. this disorder consists of clonal proliferation of megakaryocytes and granulocytic precursors in the bone marrow, accompanied by reactive, marrow fibrosis thought to be secondary to local release of fibrogenic growth factors. Replacement of the marrow by fibrosis leads to extramedullary hematopoiesis, which is most common in the

spleen and liver[111]. PMF has a prefibrotic phase, which is commonly asymptomatic and is characterized by a hypercellular marrow and minimal fibrosis. Progression leads to extensive bone marrow fibrosis. Occurring in the elderly, idiopathic myelofibrosis portends a shortened life expectancy with an increased risk of developing acute leukemia. the incidence of PMF in the US was 0.21 per 100,000 and the median age is about 67 years. 3.9%-20% patients with PMF transform into acute leukemia[112].

PMF is an infrequent disease that usually affects elderly people. Currently, median survival approaches 6 years, but there is a wide variability, ranging from less than 1 year to more than 2 decades[89].

In the 2008 WHO Diagnostic Criteria for PMF are inTable 11[103]:

	PMF
Major criteria	1. Megakaryocyte proliferation and atypia associated either with reticulin and/or collagen fibrosis, or in the absence of reticulin fibrosis, the megakaryocyte changes must be associated with increased bone marrow cellularity, granulocytic proliferation and often decreased erythropoiesis (i.e., prefibrotic disease). 2. Not meeting WHO diagnostic criteria for PV, CML, MDS or other MPN. 3. Presents of *JAK2*V617For other clonal markers or in the absents of these no evidence of reactive bone marrow fibrosis.
Minor criteria	1. Leukoerythroblastosis. 2. Increased serum lactate dehydrogenase level. 3. Anemia. 4. Splenomegaly.

Table 11: (*From Tefferi A, Leukemia. 2008 Jan; 22(1):14-22)*

The diagnosis of PMF requires meeting all 3 major criteria and 2 minor criteria.

1.4 Molecular pathogenesis in classic MPNs

1.4.1 JAK2 - STAT5 Pathway

The Janus kinase 2 (JAK2) gene was first cloned in 1989 and is

a member of the Janus family of cytoplasmic non-receptor tyrosine kinases, which also includes JAK1, JAK3 and TYK2, and transduce cytokine-mediated signals via the JAK-STAT pathway. The JAK kinases have seven homologous regions (JH1-7) named JAK homology domains[113]. At the carboxyl terminus, there are two highly phosphate transferring homologous domains: an active tyrosine kinase domain, JAK homology 1(JH1), and a catalytically inactive pseudokinase domain, JAK homology 2 (JH2), these domain is a negative regulator of JH1 domain. The JH3 and JH4 domains are called SRC homology 2 domain (SH2). At the N-terminus, JH5 and JH7 domains contain an amino terminal FERM (4-point-1, Erzin, Radixin, Moesin) homology domain where binding to type 1 cytokine receptors takes place (Figure 12)[114]. The interactions of the JAK2 FERM domain also comprises a role in trafficking of the EPO receptor (EPOR) cytoplasmic domain to the cell surface[115,116].

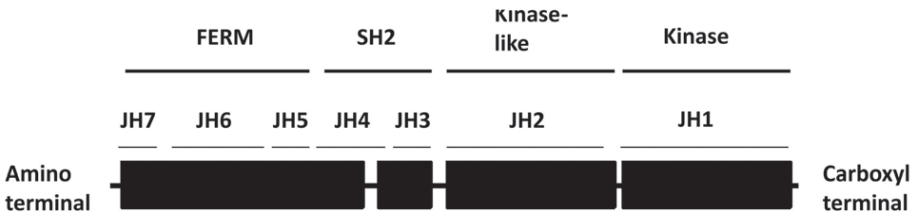

Figure 12. JAK2 gene. (*From Barcelos MM; Rev Bras Hematol Hemoter; 2011; 33 (4):290-6)*

In this context, JAK2 is frequently required for the function of a variety of cytokine receptors, including all homodimeric receptors like EPO-R, TPO (trombopoietin receptor), and some heterodimeric like receptor IL-3 (Interleukin 3), IL-5 (Interleukin 5) GM-CSF R

(Granulocyte macrophage colony stimulating factor receptor), or interferon gamma (IFNγ).

The JAK/STAT pathway is the principal signaling mechanism for a wide array of cytokines and growth factors. JAK activation stimulates cell proliferation, differentiation, cell migration and apoptosis. These cellular events are critical to hematopoiesis, immune development, mammary gland development and lactation, adipogenesis, sexually dimorphic growth and other processes[115].

Intracellular activation occurs when ligand binding induces the multimerization of receptor subunits. For some ligands, such as erythropoietin, the receptor subunits are bound as homodimers while, for others, such as interferons and interleukins, the receptor subunits are heteromultimers. For signal propagation through either homodimers or heteromultimers, the cytoplasmic domains of two receptor subunits must be associated with JAK tyrosine kinases. JAKs are distinctive in that they have tandem kinase-homologous domains at the C-terminus. The first is a non-catalytic regulatory domain, whereas the second has tyrosine kinase activity, as seen above (Figure 13)[117]. JAK activation occurs upon ligand-mediated receptor multimerization because two JAKs are brought into close proximity, allowing trans-phosphorylation[115].

This primarily leads to the recruitment of STAT (signal transducer and activator of transcription) molecules which are then phosphorylated, STAT are latent transcription factors that reside in the cytoplasm until activated, the seven mammalian STATs bear a conserved tyrosine residue near the C-terminus that is phosphorylated by JAKs. This phosphotyrosine permits the dimerization of STATs through interaction with a conserved SH2

domain. Phosphorylated STATs enter the nucleus, once in the nucleus, dimerized STATs bind specific regulatory sequences to activate or repress transcription of target genes. Thus the JAK/STAT cascade provides a direct mechanism to translate an extracellular signal into a transcriptional response (Figure 13)[115].

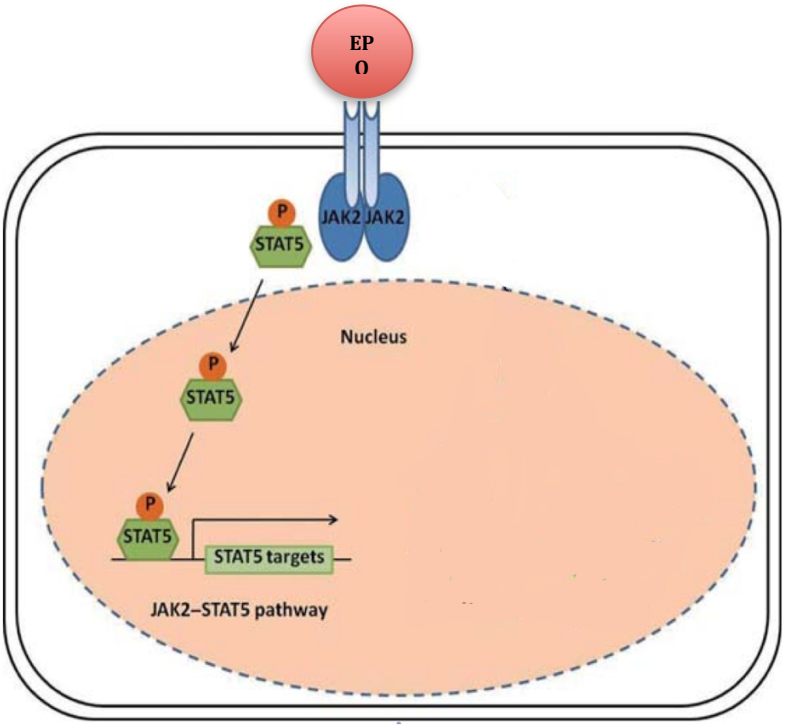

Figure 13. JAK2-STAT5 pathway. JAK2 is activated by phosphorylation upon the association of receptors and their ligands. The active JAK2 further phosphorylates the STAT5, which is translocated into the nucleus to activate its target genes. *(Adapted from He J; J Mol Cell Biol; 2010 Oct; 2 (5):231-3)*

1.4.2 JAK2 ^{V617F} mutation in MPNs

In 2005, several independent groups used different genetic, genomic and functional approaches, identified the point mutation in

JAK2 in most patients with PV, ET or PMF. To mention few, William Vainchenker's group found that EEC formation was abolished by a pharmacological inhibitor of JAK2 as well as an siRNA against JAK2[100]. Levine's group used an high-throughput DNA sequence analysis and they hypothesized that activating mutations in tyrosine kinases are important in the pathogenesis of PV, ET, and PMF[98]. Chloe´ James and colleagues as well as Joanna Baxter and colleagues described a clonal and recurrent mutation in the JH2 pseudo-kinase domain of the Janus kinase 2 (JAK2) gene in most of PV patients[99]. Robert Kralovics and collegues characterized and sequenced by genome-wide screening, the genes in the minimum region affected by acquired uniparental disomy of chromosome 9p involved in loss of heterozygosity (LOH) in PV[118].

The mutation is a guanine to thymidine substitution at nucleotide 1849, in exon 14, which results in a substitution of valine to phenylalanine at codon 617 of JAK2 ($JAK2^{V617F}$) (Figure 14)[119], these mutation is present in hematopoietic cells but not germline DNA in patients with MPD, demonstrating that $JAK2^{V617F}$ is a somatic mutation that is acquired in the hematopoietic compartment.

The $JAK2^{V617F}$ protein has constitutive kinase activity, and when expressed *in vitro* $JAK2^{V617F}$, but not wild-type JAK2, is constitutively phosphorylated, which is consistent with the notion that $JAK2^{V617F}$ is a gain-of-function mutation with respect to JAK2 kinase activity. Expression of $JAK2^{V617F}$ confers cytokine hypersensitivity and cytokine-independent growth to hematopoietic cells, which are characteristic features of hematopoietic colonies grown from patients with PV and can activate the downstream pathways including JAK2/STAT5, ErK1/2 MAPK and PI3K/AKT in

the absence of cytokines[120,121].

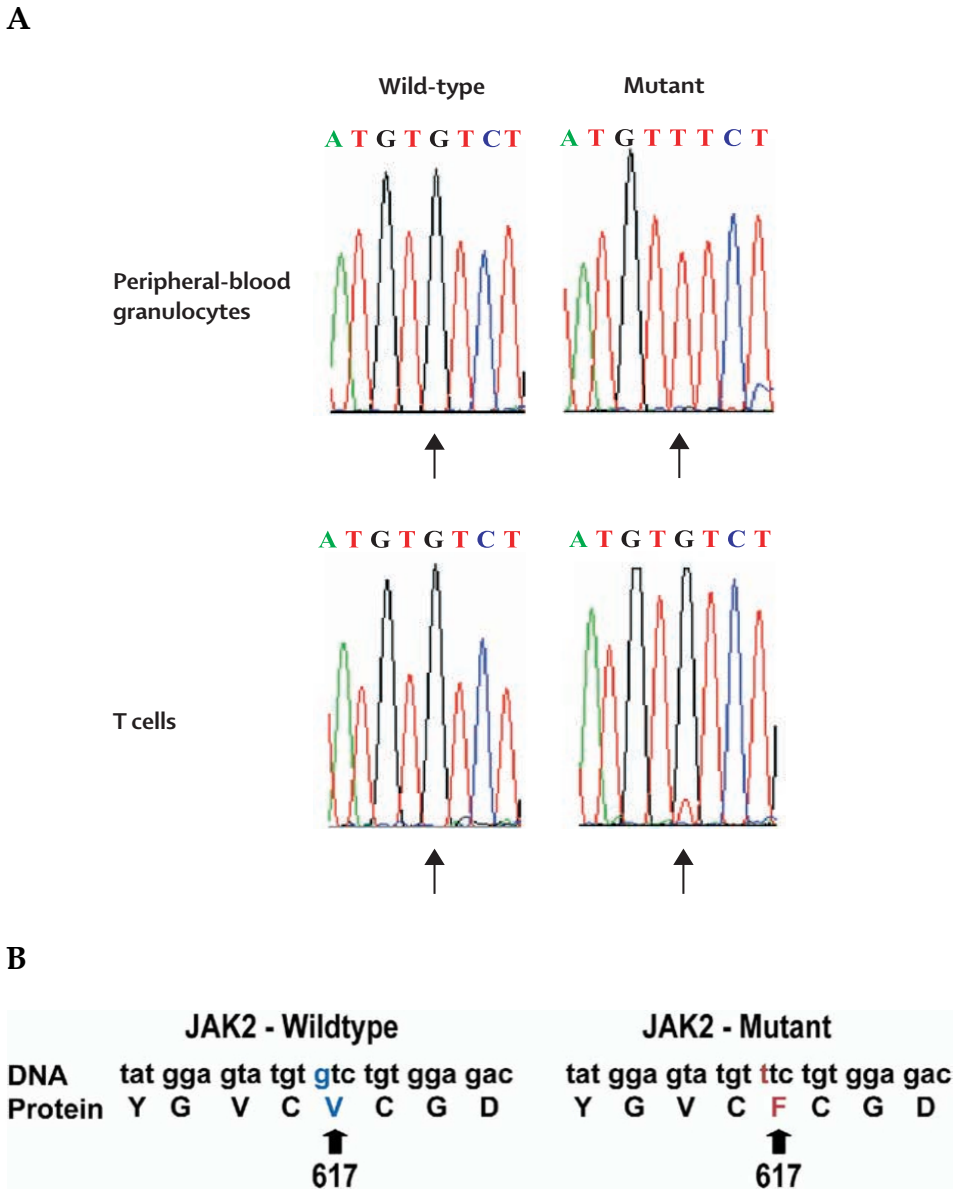

Figure 14. The *JAK2* Mutation in Patients with MPN. Panel A shows a G - T transversion in *JAK2* (arrow) in a patient with the mutation (right). The

mutation was present in DNA from granulocytes, but absent in T cells, consistent with the existence of an acquired somatic origin of the mutation. Panel B shows DNA sequence and protein translation for both the wild-type and mutant *JAK2* alleles. The guanine to thymine substitution results in a valine to phenylalanine substitution at codon 617. (*Adapted From Baxter EJ; Lancet; 2005 Mar 19-25; 365(9464):1054-61 and Levine RL, Cancer Cell, 2005 Apr; 7 (4):387-97)*

The discovery of $JAK2^{V617F}$ mutation has undoubtedly revolutionised the diagnosis and provided a tremendous advance in the pathogenesis of classic chronic MPNs, and sesitive, allele-specific assays have been used to assess the frequency of $JAK2^{V617F}$ mutations in different malignancies and to a lesser degree, in a number of other myeloid malignancies such as CML, myelodysplasia (most common with refractory anaemia with ringed sideroblasts with thrombocytosis), and AML (Table 12)[122-124]. Detection of this mutation is beneficial in differentiating between a reactive hematological response and a true clonal disorder and can also serve as a target for therapeutic intervention.

Disease	Frequency
Polycythaemia Vera	81-99%
Essential Thrombocytosis	41-72%
Primary Mielofibrosis	39-57%
Chronic Myelomonocytic Leukemia	3-9%
Myelodysplasia (ringed sideroblast and thrombocytosis)	3-5%
Acute Myeloid Leukemia	< 5%

Table 12. Frequency of the $JAK2^{V617F}$ allele in myeloid disorders. (*Adapted From Levine RL; Nat Rev Cancer; 2007 Sep;7(9):673-83)*

Biochemical studies have shown that the JAK2^{V617F} mutation causes cytokine-independent activation of JAK–STAT, PI3K, and AKT (also known as protein kinase B) pathways, and mitogen-activated protein kinase (MAPK) and extracellularsignal-regulated kinase (ERK), all of which are implicated in erythropoietin-receptor signaling (Figure 15)[123,125].

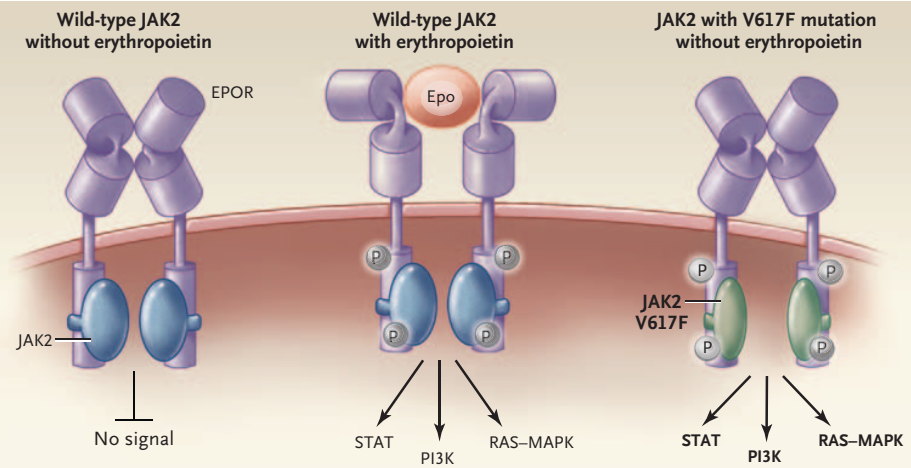

Figure 15. Role of JAK2 in Pathway Signaling. In the absence of ligand, the erythropoietin receptor binds JAK2 as an inactive dimer. In cells with wild-type JAK2 protein, the binding of erythropoietin to its receptor induces conformational changes in the receptor, resulting in phosphorylation (P) of JAK2 and the cytoplasmic tail of the receptor. This leads to signaling through pathways made up of JAK–STAT, phosphatidylinositol 3 kinase (PI3K), and RAS and mitogen-activated protein kinase (RAS–MAPK). In cells with the V617F mutation, the signaling is constitutively increased, even in the absence of erythropoietin. (*From Campbell PJ.; N Engl J Med; 2006 Dec 7; 355(23):2452-66)*

These activation of multiple down-stream signaling pathways,

effects on gene transcription, apoptosis, the cell cycle, and differentiation. Effects on apoptosis include overexpression of the cell-survival protein BCL-X in erythroid precursor cells in polycythemia vera, probably as a result of enhanced JAK– STAT signaling[126,127]. With respect to the cell cycle, mutant JAK2 promotes G1/S phase transition in hematopoietic cell lines. Effects on erythroid differentiation may be mediated by nuclear factor erythroid-derived 2 (NF-E2)[128], which is up-regulated in PV and plays an important role in erythroid differentiation[129,130].

1.5 Scope of the thesis

The aims of my PhD thesis project were to study the mechanisms of action of GVS in MPN, more in particular to analyse the biological effects of low and high doses of GVS on JAK2^{V617F} mutated cells in long and short terms assays (proliferation, cell cycle, apoptosis), and to study the early modifications of the gene expression profile in JAK2^{V617F} cells using microarrays. Moreover I wished to investigate in vitro the activity of HDACi with other drugs like hydroxyurea (HU), and to propose new approaches to optimize their therapeutic efficacy.

The project has developed as follows:

- The first part of the project has the purpose to analyse the biological effects of low and high doses of GVS on JAK2^{V617F} mutated cells in long and short terms assays (proliferation, cell cycle and apoptosis), and to study the mechanisms of action of GVS in JAK2^{V617F} cells by gene expression profile.

- The second part of the project wants to evaluate the possibility to improve the anti-tumor activity of GVS by the combined use of GVS with HU, and determine whether clinically achievable concentrations of GVS and HU induce synergistic cytotoxicity in JAK2^{V617F} cells in vitro, and to investigate the possible interaction between GVS and HU at the molecular level.

1.6 References

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Chapter 2

The HDAC inhibitor Givinostat modulates the hematopoietic transcription factors NF-E2 and C-MYB in JAK2(V617F) myeloproliferative neoplasm cells.

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Abstract

We investigated the mechanism of action of the histone deacetylase inhibitor Givinostat (GVS) in Janus kinase 2 (JAK2)V617F myeloproliferative neoplasm (MPN) cells. GVS inhibited colony formation and proliferation and induced apoptosis at doses two- to threefold lower in a panel of JAK2^{V617F} MPN compared to JAK2 wild-type myeloid leukemia cell lines. By global gene expression analysis, we observed that at 6 hours, GVS modulated 293 common genes in the JAK2^{V617F} cell lines HEL and UKE1, of which 19 are implicated in cell cycle regulation and 33 in hematopoiesis. In particular, the hematopoietic transcription factors NF-E2 and C-MYB were downmodulated by the drug specifically in JAK2^{V617F} cells at both the RNA and protein level. GVS also inhibited JAK2-signal transducer and activator of tran- scription 5-extracellular signal-regulated kinase 1/2 phosphorylation, but modulation of NF-E2 and C-MYB was JAK2-independent, as shown using the JAK2 inhibitor TG101209. GVS had a direct effect on the NF-E2 promoters, as demonstrated by specific enrichment of associated histone H3 acetylated at lysine 9. Modulation by GVS of NF-E2 was also observed in freshly isolated CD34+ cells from MPN patients, and was accompanied by inhibition of their proliferation and differentiation toward the erythroid lineage. We conclude that GVS acts on MPN cells through dual JAK2-signal transducer and activator of transcription 5-extracellular signal-regulated kinase 1/2 inhibition and downmodulation of NF-E2 and C-MYB transcription.

Introduction

Philadelphia-negative myeloproliferative neoplasms (MPN) are clonal hematopoietic stem cell diseases characterized by deregulated proliferation and differentiation in several myeloid lineages. JAK2 mutations (at position 617 or within exon 12) are detected in most MPN patients of the polycythemia vera subgroup (95% of cases), and in about 50% of essential thrombocythemia (ET) and primary myelofibrosis patients [1,2]. JAK2V617F confers to hematopoietic progenitor cells growth factor_independent proliferation [3]. In addition, it induces a transplantable myeloproliferative disease resembling human polycythemia vera or ET in vivo in transgenic or knock in animals [4–8].

The pan-histone deacetylase inhibitor (HDACi) Givinostat (GVS, ITF2357) has antiproliferative and proapoptotic activity against several cancer cells, including acute myelogenous leukemia and multiple myeloma cells in vitro and in vivo [9]. Recently, GVS has been discovered to inhibit colony formation of cells from MPN patients at doses at least 10 times lower than those required to affect cells bearing wild-type (wt) JAK2 (JAK2wt) [10]. Furthermore exposure to low doses of GVS allows the preferential expansion of JAK2wt over JAK2 mutated cells in colony assays using MPN patients' cells [10]. The mechanism for the higher sensitivity to GVS of JAK2V617F cells is unclear, although the drug has been observed to downmodulate phosphorylated JAK2 and signal transducer and activator of transcription-5 (STAT-5) in MPN cells [10]. These findings have led to the initiation of a phase I/II clinical

studies of the drug, alone or in combination with hydroxyurea, in JAK2^{V617F} MPN patients [11] (www.clinicaltrials.gov).

In this report, we analyzed in more detail the biological activities of GVS in JAK2^{V617F} cells using a panel of JAK2^{V617F} or JAK2wt cell lines [12], as well as CD34^{+} cells isolated from MPN patients, measuring, in particular, proliferation, apoptosis, and differentiation along the erythroid lineage. By global gene expression profiling (GEP), we demonstrate that GVS rapidly modulates important hematopoietic transcription factors (TF), including C-MYB and, most interestingly NF-E2, a TF overexpressed in MPN and crucial for erythromyeloid lineage differentiation [13–15].

Materials and methods

Drugs and cell lines

The pan-HDAC inhibitor GVS (ITF2357) was a kind gift from Italfarmaco, Milano, Italy [10]. The JAK2 inhibitor TG101209 was from Axon Medchem (Groningen, The Netherlands).

The erythroleukemia cell line HEL, the ET cell line SET2, and the chronic myeloid leukemia cell line KU812 were from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The ET cell line UKE1 was a kind gift of Dr. Walter Fiedler (Eppendorf Hospital, Hamburg, Germany) [16]. The chronic myeloid leukemia cell line K562 and acute myelogenous leukemia cell line KG1 have been described previously [10]. HEL, UKE1, and SET2 all bear the JAK2V617F mutation [12], as confirmed by polymerase chain reac- tion (PCR) in our laboratory (data not shown). K562 and KU812 carry the bcr-abl translocation. Cell lines were maintained in RPMI-1640 medium (Lonza, Basel, Switzerland) supplemented with 2 mM glutamine (Euroclone, Wetherby, West Yorkshire, UK), 110 mM gentamycin (PHT Pharma, Milano, Italy), and 10% fetal calf serum (HEK, K562, KG1; Euroclone), 20% fetal calf serum (SET2, KU812) or, for the UKE1 cell line, 10% fetal calf serum plus 10% horse serum (GIBCO Invitrogen Corp., Paisley, UK), 1 mM hydrocortisone, and 25 mM HEPES buffer solution (Sigma-Aldrich, Milan, Italy).

Peripheral blood from MPN patients was obtained after informed consent as approved by the Internal Ethical Committee. CD34+ cells were purified to >70% purity by antibody-based magnetic bead separation (Miltenyi, Bergsch Gladbach, Germany).

Colony-forming assay

Five-thousand cells were plated in methylcellulose medium (HSC- CFU containing recombinant human erythropoietin [rh-EPO; Mil- tenyi Biotech]) for 10 days at 37_C 5% CO2 [10].

Cytotoxicity assays

Cytotoxicity assays were performed using the alamar blue vital dye (AbD Serotec, Kidlington, UK), as described [10].

Proliferation and cell death

Cells were cultured at 0.5–1 105 cell/mL in complete medium and absolute cell number and cell death were determined at different times by flow cytometry using calibration beads (Bright Count Microspheres; IQ Products, Groningen, The Netherlands) and 7-aminoactinomycin D (BD Biosciences, Buccinasco, Italy), respectively, on a FACSCalibur Instrument (BD Biosciences).

Erythroid differentiation

$CD34^+$ cells were cultured at 7 104 cell/mL in StemSpan Serum- Free Expansion Medium (Stem Cell Technologies, Vancouver, BC, Canada) supplemented with 50 ng/mL recombinant human stem cell factor (rhSCF), 1 IU/mL rhEPO, 10 ng/mL rh interleukin-3 (all from Sigma Aldrich), 40 ng/mL human low-density lipoprotein (Stem Cell Technologies). Absolute total and $CD34^+$ cell numbers were determined at different times by single-platform flow cytometric $CD34^+$ cell count analysis using CD34-phycoerythrin and CD45-fluorescein isothiocyanate antibodies (BD Biosciences)

according to the guidelines of the International Society of Hematotherapy and Graft Engineering [17]. For evaluation of erythroid differentiation, cells were stained with phycoerythrin-conjugated anti-CD235a/GpA and allophycocyanin-conjugated anti-CD36 antibodies (BD Biosciences) and analyzed on a FACScan (BD Biosciences).

Western blotting

Cells were lysed in M-PER extraction reagent (Pierce, Rockford, IL, USA). Equivalent amounts of protein were analyzed by stan- dard Western blotting. The following antibodies were used: JAK2, STAT5, TAL1, and NF-E2 (Santa Cruz Biotechnology, Inc., Heidelberg, Germany); phosphorylated JAK2 (Tyr1007/1008) and STAT5 (Tyr694) (Cell Signaling Technology, Danvers, MA, USA), and c-myb clone CB10018 [18]. Detection was performed using horseradish peroxidase_labeled secondary antibodies (Santa Cruz) and Super Signal West Pico Chemiluminescent Substrate (Pierce).

Gene expression profiling

HEL and UKE1 cells were cultured in presence or absence of 250 nM GVS in triplicate flasks for 6 hours and lysed in TRIzol Reagent (Life Technologies, Inc., Rockville, MD, USA). Total RNA was purified from each using the RNeasy total RNA Isolation Kit (Qiagen, Valencia, CA, USA). Preparation of DNA single-stranded sense target, hybridization to GeneChip Gene 1.0 ST Array (Affymetrix Inc., Santa Clara, CA, USA) and scanning of the chips (7G Scanner; Affymetrix Inc.) were carried out according to manufacturer's protocols. Signal intensities were converted to

expression values by probe set summarization, robust multiarray average normalization, and log2 transformation procedures using the Expression Console Software (Affymetrix Inc.). Probe sets mapping on the same gene (according to the hugene- 1_0-st-v1_na30_hg19 Affymetrix Annotation file) were considered as a single series of expression values by median summarization, resulting in 20,072 unique genes.

The gene expression analyses were performed using the RankProd R package (http://www.bioconductor.org/) [19]. RankProd utilizes the nonparametric method rank product to identify upregulated or downregulated genes. The algorithm detects genes consistently highly ranked in a two-condition experiments comparison [19]. Two statistical filters on percentage of false-positive predictions (pfp < 0.05) and on the difference in gene expression values (fold change >_2) were applied to the ranked list of genes. Only the matching list (293 genes) of the differentially expressed genes in both HEL and UKE1 was considered for functional annotation.

The functional analysis of the selected genes was performed by means of NetAffx (Affymetrix at https://www.affymetrix.com/ analysis/netaffx/) and the Database for Annotation, Visualization and Integrated Discovery Tool 6.7 (http://david.abcc.ncifcrf.gov/). [20,21]. The Functional Annotation Clustering tool of Database for Annotation, Visualization and Integrated Discovery measures relationships among the annotation terms in order to group the similar and redundant annotation contents from different sources into a single annotation cluster. The Enrichment Score for each annotation term is calculated as the minus log geometric mean of all the enrichment p values associated with the

gene members included in each group. Only the Gene Ontology Biological Process and Molecular Function terms were chosen as annotation categories and ''medium'' classification stringency was set for the analysis. The clusters with an Enrichment Score O1.3 (equivalent to an average p value of 0.05) [20, 21] were selected among the 65 annotation clusters globally identified.

Real-time PCR

Total RNA was extracted from three independent experiments, different from those used for microarray, using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). Complementary DNA was synthesized using the SuperScript III First-strand Synthesis System for reverse transcription PCR (Invitrogen). PCR was performed with specific primers (Supplementary Table E1; online only, available at www.exphem.org) on the LightCycler 2.0 Instruments (Roche Diagnostics, Indianapolis, IN, USA) using LightCy- cler FastStart DNA MasterPLUS SYBR Green I mix (Roche Diagnostics) and the RPL13A ribosomal gene as the normalization control.

Relative quantification was performed using the comparative Ct (2-ΔΔCt) method, where ΔΔCt 5 mean DCt gene – mean ΔCt housekeeping.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed as described previously [22]. Briefly, cross-linked chromatin from treated and untreated UKE1 and SET2 cells was sonicated and immunoprecipitated with 3 mg of antibodies specific

for the following: histone H3 either unmodified (H3; Abcam, Cambridge MA) or acetylated at lysine 9 (H3K9ac) or 14 (H3K14ac) (both from Active Motif, La Hulpe, Belgium) and Thioredoxin as control (Genespin, Milan, Italy). The primers used to amplify NF-E2 promoters, control AGTR1 promoter and satellite centromeric region (SAT_CEN) are listed in Supplementary Table E1 (online only, available at www.exphem.org). We perform control amplifications of centromeric regions as a routine step to verify that adequate immunoprecipitation has taken place. The satellite sequences are amplified from the immunoprecipitates obtained with anti-histone H3 antibody, but not anti-acetylated histone anti-bodies, because histones are not significantly acetylated in these satellite regions [23]. RTQ-PCR was performed using SYBR Green Buffer (Genespin) in the iCycler IQ detection system (Bio-Rad, Hercules, CA, USA). The relative sample enrichment, normalized at first on a satellite centromeric region, was calculated with the following formula: 2DCtx _ 2DCtb, where DCtx 5 Ct input _ Ct sample, and DCtb 5 Ct input _ Ct control antibody (TRX). Each value of acetylation of lysine 9 and 14 has been normalized on the total amount of immunoprecipitated unmodified H3 [24].

Chromatin immunoprecipitation

Differences between treated and untreated or mutated and wt cell lines were analyzed by unpaired Student's t test. Colony assay curves were analyzed using the nonparametric Mann-Whitney test. The effect of GVS during time on proliferation and differentiation was analyzed by analysis of variance for repeated measures.

Results

JAK2-mutated cell lines are more sensitive to GVS than JAK2wt cell lines

We have previously shown that the JAK2V617F-positive cell line HEL (homozygous) showed an IC50 in response to GVS in cytotoxicity assays about threefold lower than JAK2wt myeloid cell lines [10]. In order to extend these observations to a larger panel of cell lines, we analyzed the effect of the drug on UKE1 (homozygous) and SET2 (heterozygous) in alamar blue assays. As shown in Figure 1A and B, the JAK2-mutated cell lines responded to GVS with an IC50 of 60 to 100 nM, whereas an approximately threefold higher IC50 was observed with JAK2wt cell lines (KU812, THP1, and K562) ($p < 0.001$; Fig. 1B). Similarly in colony assays, the response to GVS of HEL and UKE1 (JAK2V617F) was significantly different than that of K562 and THP1 (JAK2wt) with ED50 of about 40 nM vs 80 to 220 nM, respectively ($p < 0.01$; Fig. 2A and B). The difference was significant also when each JAK2V617F cell line was compared singly to each JAK2wt line ($p < 0.05$). The other two cell lines did not form clear colonies (data not shown).

We next wondered whether the different sensitivity to GVS was at the level of proliferation or apoptosis. Live cell counts at different time points showed that GVS started to inhibit expansion of the UKE1 and SET2 cell lines already at 50 nM, and of HEL at 100 nM, whereas a 250 nM dose was required to inhibit to a similar extent K562, KU812, or THP1 cells (Supplementary Figure E1; online only,

available at www.exphem.org). These data were confirmed in standard cell cycle experiments, showing a 3% to 5% reduction in S-phase cells and 6% to 7% increased G1 phase cells after 48 hours treatment in HEL and UKE1 cells treated with 100 nM GVS ($p < 0.05$), and no significant change observed in the K562, THP1, and KG1 cell lines at the same dose (Supplementary Figure E2; online only, available at www.exphem.org).

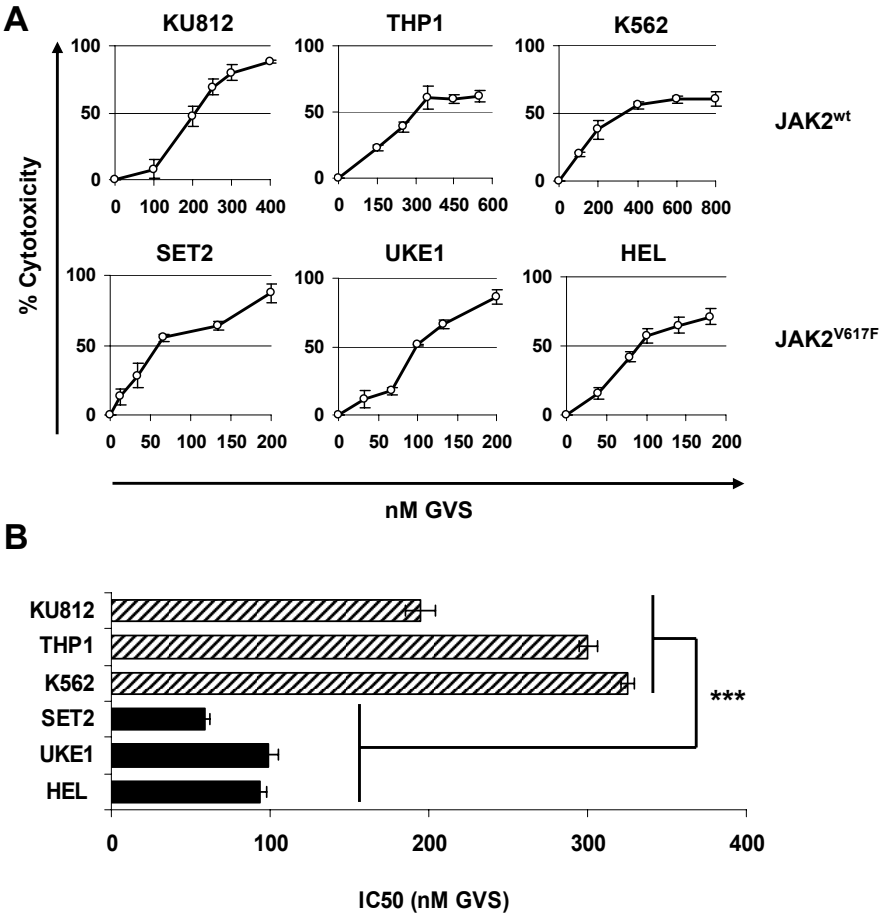


Figure 1. JAK2V617F cell lines are more sensitive to GVS than JAK2wt cells in cytotoxicity assays. The indicated JAK2wt or JAK2V617F cell lines were treated with GVS (1 nM to 10 mM) and alamar blue added 48 hours

later. (A) Data shown are the means and standard deviations of three independent dose_response experiments for each cell line. (B) Mean IC50 and standard deviations obtained from three independent experiments with JAK2wt (striped bars) or JAK2V617F cell lines (black bars) are shown. ***p < 0.001.

As far as apoptosis is concerned, 100 nM and 250 nM GVS induced more apoptosis of JAK2V617F than wt cells at days 3 and 1, respectively (Fig. 3A and B). The difference was statistically significant when the JAK2V617F vs JAK2 wt cell lines were globally compared with each other ($p < 0.01$ and < 0.001 at day 3 and 1, respectively).

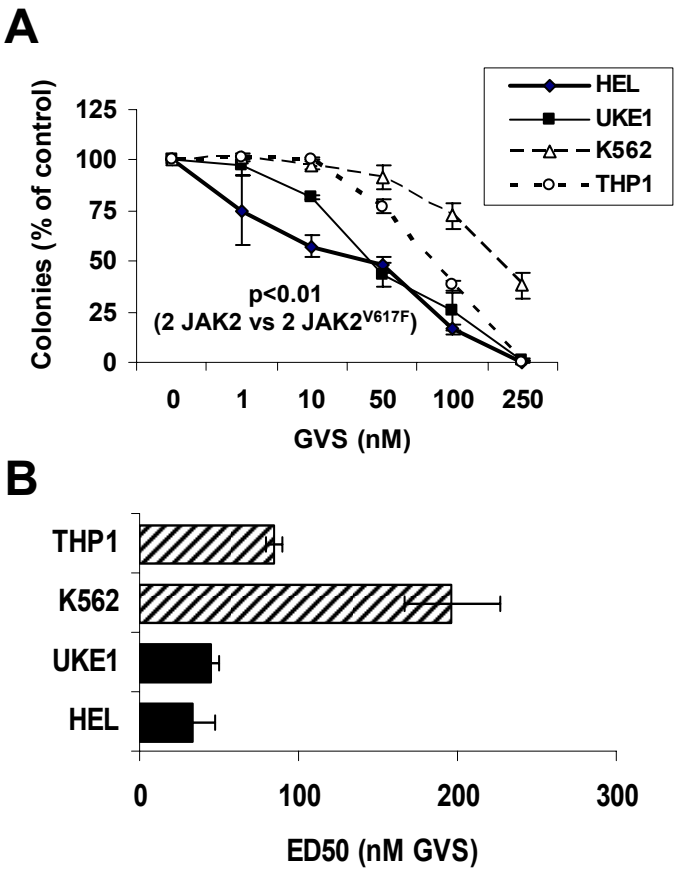


Figure 2. Cell lines bearing JAK2V617F are more sensitive to GVS than JAK2wt cells in colony assays. The indicated cell lines were plated in methylcellulose in the absence or presence of increasing concentrations of GVS (1_250 nM) and colonies counted after 10 days. (A) The dose_ response curves for each cell line and (B) ED50 values for each JAK2wt (striped bars) or JAK2V617F cell lines (black bars). Results are the mean percentages and standard deviations of treated relative to untreated controls from three independent experiments for each cell line.

Differences were also significant when the single JAK2V617F cell lines were compared with each JAK2wt line (p at least < 0.05, except for HEL vs KG1 at day 1, where the difference was not statistically significant) (Supplementary Table E2; online only, available at www.exphem.org). The original dot plots and histograms of a representative experiment are shown in Supplementary Figure E3A and B (online only, available at www.exphem.org).

Global gene expression profiling in JAK2V617F cell lines

In order to analyze the mechanism of action of GVS in JAK2V617F cells at a molecular level, we performed GEP. The HEL and UKE1 cell lines were cultured in triplicate flasks for 6 hours in presence or absence of 250 nM GVS. RNA was extracted and probes prepared for hybridization to the Affymetrix GeneChip Gene 1.0 ST array (Affymetrix) representing >20,000 human genes. Genes significantly up-or downmodulated at least twofold were identified using the nonparametric rank product method. Seven hundred and sixteen genes were modulated by the drug in HEL (258 down and 458 up) and 863 genes in UKE1 (430 down and 433 up). A total of common 293 genes were modulated in both cell lines, of which 114

down and 179 up and these are listed in Supplementary Table E3 (online only, available at www.exphem.org). The complete microarray original data have been deposited in the Gene Expression Omnibus repository (GSE27615).

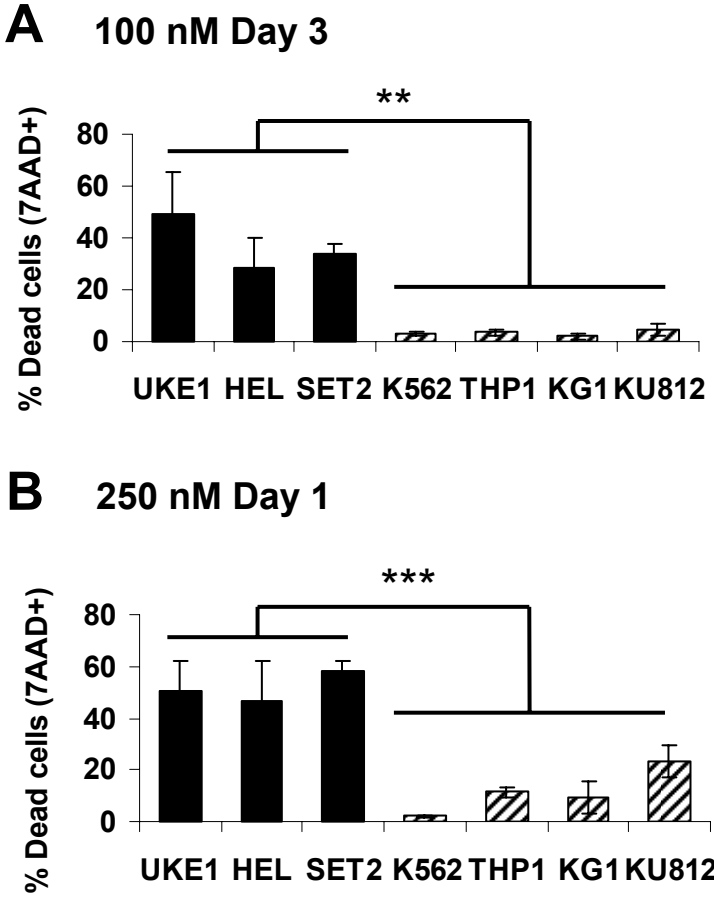


Figure 3. The JAK2V617F cell lines are more sensitive to GVS than JAK2wt cells in apoptosis assays. The indicated JAK2V617F (black bars) or JAK2wt cell lines (striped bars) were plated in absence or presence of 100 nM (A) or 250 nM GVS (B). After 1 day (B) or 3 days (A), the percentage of 7-aminoactinomycin D + cells was measured by flow cytometry. Results

shown are the means and standard deviations of three independent experiments for each cell line.

Hierarchical clustering analysis was performed on the 293 common genes modulated by GVS at 6 hours, revealing cell cycle as the functional category with the highest enrichment score (1.48) (Supplementary Table E4; online only, available at www.exphem.org), with 19 genes belonging to this category. The single cell cycle_associated genes are listed in Supplementary Figure E4 (online only, available at www.exphem.org). Of particular interest is the induction of RGS2, CCNG2 (cyclin G2), NOTCH2, and MLF1 and downmodulation of HCFC1, all of which can contribute to the inhibition of proliferation. Other annotation clusters included 14 genes involved in cellular amino acid and derivative metabolic process (1.42 enrichment score) and 7 genes related to methyltransferase activity (1.39 enrichment score) (Supplementary Table E4; online only, available at www.exphem.org).

Given the cellular context under study, we specifically searched, among the 293 commonly modulated genes, those known to play a role in hematopoiesis. We found 33 modulated genes that are directly or indirectly relevant for hematopoiesis and these are listed in Figure 4A. They include 8 genes related to migration and adhesion: CX3CR1, sphingosine-1 phosphate lyase 1 (involved in lymphocyte egress from thymus), STAT4 (a TF downstream from CXCR4), cyclic adenosine monophosphate response element binding protein 3 (implicated in monocyte migration), all upregulated by GVS, as well as selectin P ligand, chemokine (C-X-C motif) ligand 2, CD84 (SLAM family member 5), and CD244 (natural killer cell receptor 2B4), all downmodulated by the drug. The other 25

hematopoietic genes included many TF and signaling molecules. In order to further define which genes might be of major interest in our cellular context, on the basis of an extensive search of the literature, we were able to locate 23 of these 25 genes in specific points along the hematopoietic differentiation dendogram, as illustrated in Figure 4B. Most of these genes are thought to be involved directly or indirectly in the control of either multipotent stem cells, the erythrothrombopoietic-myeloid lineage, the lymphoid lineage, or in some cases in more than one differentiation steps (Fig. 4B)

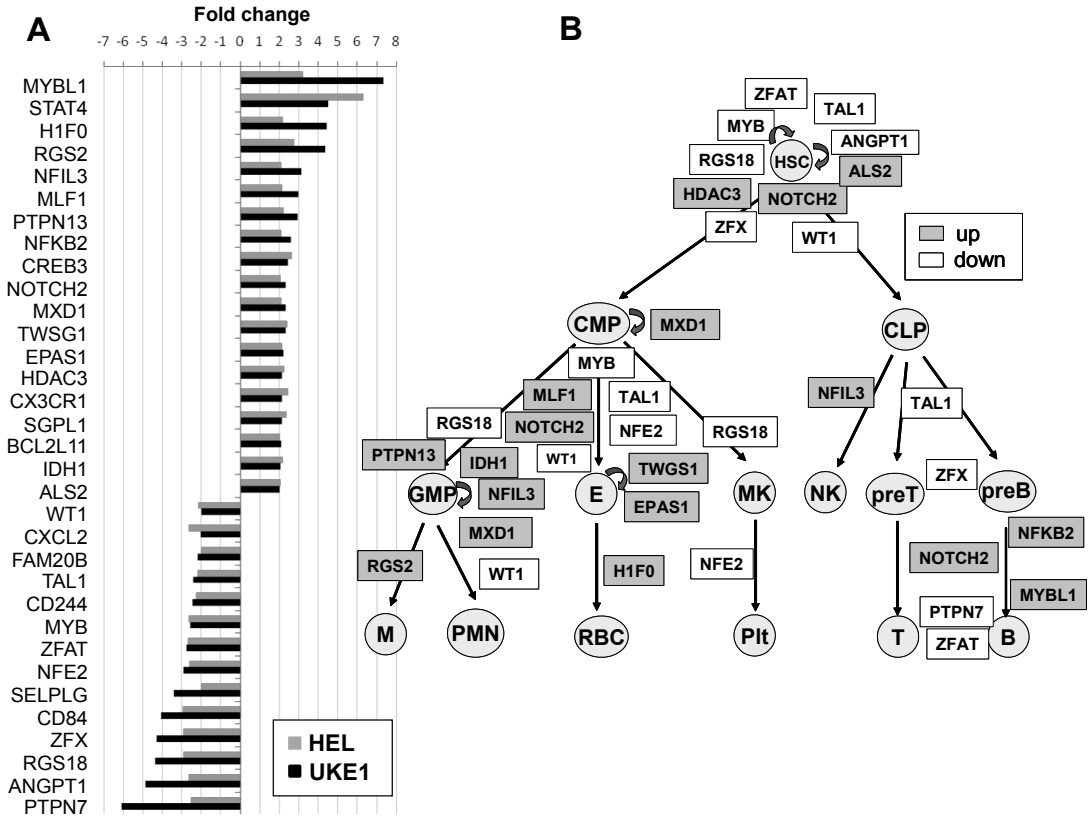


Figure 4. GVS modulates 33 genes involved in hematopoiesis. (A) The 33 hematopoiesis-associated genes modulated by GVS in both HEL (gray bars)

and UKE1 (black bars) are shown. (B) The major steps along the hematopoiesis differentiation dendogram where 25 of the GVS modulated genes are known to be involved are indicated. B 5 mature B cell; CLP 5 common lymphoid progenitor; CMP 5 common myeloid progenitor; E 5 erythroid progenitor; GMP 5 granulocyte-macrophage progenitor; M 5 monocyte; MK 5 megakaryocyte; NK 5 natural killer cell; Plt 5 platelet; PMN 5 polymorphonuclear phagocyte; PreT 5 T-cell precursor, preB 5 B-cell precursor; RBC 5 red blood cell; T 5 mature T cell.

GVS downmodulates NF-E2 and C-MYB proteins.

A set of seven genes was selected for further validation by real-time quantitative (RTQ) PCR (downmodulated NF-E2, C-MYB, and TAL1 and upregulated NOTCH2, CCNG2, MXD1, and NFKB2). For all seven genes analyzed, the RTQ-PCR confirmed the modulation detected by microarray, thus fully validating the GEP analysis (Fig. 5A).

Given the importance of NF-E2, C-MYB, and TAL1 in regulating differentiation along the erythroid, megakaryocytic, and myeloid lineages (Fig. 4B), and/or in MPN [13–15, 25], these TF were selected for further analysis at the protein level. GVS significantly downmodulated NF-E2 and C-MYB proteins by 60% to 90% in all three JAK2- mutated cell lines after 24 hours treatment, in agreement with messenger RNA expression data (Fig. 5B). In contrast, little downmodulation of TAL1 protein could be detected in the same cell lines (Fig. 5B).

We then investigated whether TF modulation was specific for JAK2V617F cells or could be observed also in JAK2wt cells. As shown in Figure 5B, NF-E2 was, if anything, induced in K562, THP1, and

KU812. C-MYB was moderately downmodulated (by about 50%) in K562 and slightly upregulated in THP1 and KU812.
In summary, seven genes were validated by RTQ-PCR and NF-E2 and C-MYB were found to be specifically downmodulated in JAK2V617F cells at the protein level.

TF modulation by GVS is independent from the JAK2-STAT5 pathway.

We have shown previously that GVS inhibits phosphorylation of JAK2 and downstream STAT5 in HEL cells [10]. We extended these findings to other JAK2-mutated cell lines and to extracellular signal-regulated kinase (ERK)1/2, another known target of JAK2. We could confirm that GVS inhibited by 70% to 100% expression of phospho-JAK2 and by 80% to 100% downstream phospho-STAT5 and phospho-ERK1/2 in all three JAK2V617F cell lines, without significantly affecting total protein levels (Fig. 5C) [10].

Interestingly, JAK2 phosphorylation was also strongly inhibited by the drug in K562, was induced in THP1, and undetectable in KU812. STAT5 and ERK1/ 2 phosphorylation, however, were not significantly downmodulated in any of the JAK2wt cell lines (Fig. 5C). GVS inhibits significantly the JAK2-STAT5-ERK1/2 pathway in all mutated cell lines, but it does not inhibit downstream STAT5-ERK1/2 phosphorylation in any of the JAK2wt cells.

Given the strong inhibition of JAK2, STAT5, and ERK1/2 phosphorylation induced by GVS in JAK2V617F cells, we hypothesized that some of the genes modulated by the drug may be targets of the JAK2-STAT5-ERK1/2 pathway. In order to test this

hypothesis, we used the selective JAK2 inhibitor TG101209. As expected, JAK2, STAT5, and ERK1/2 phosphorylation were fully inhibited by both

TG101209 and GVS in SET2 and UKE1, and total STAT5 and ERK1/2 proteins were not significantly affected in the same conditions (Fig. 5D). Among the TF analyzed, NF-E2 and C-MYB were downmodulated only by GVS and not by JAK2 inhibitor in both cell lines (Fig. 5D), showing that modulation of these TF by GVS is largely JAK2 independent.

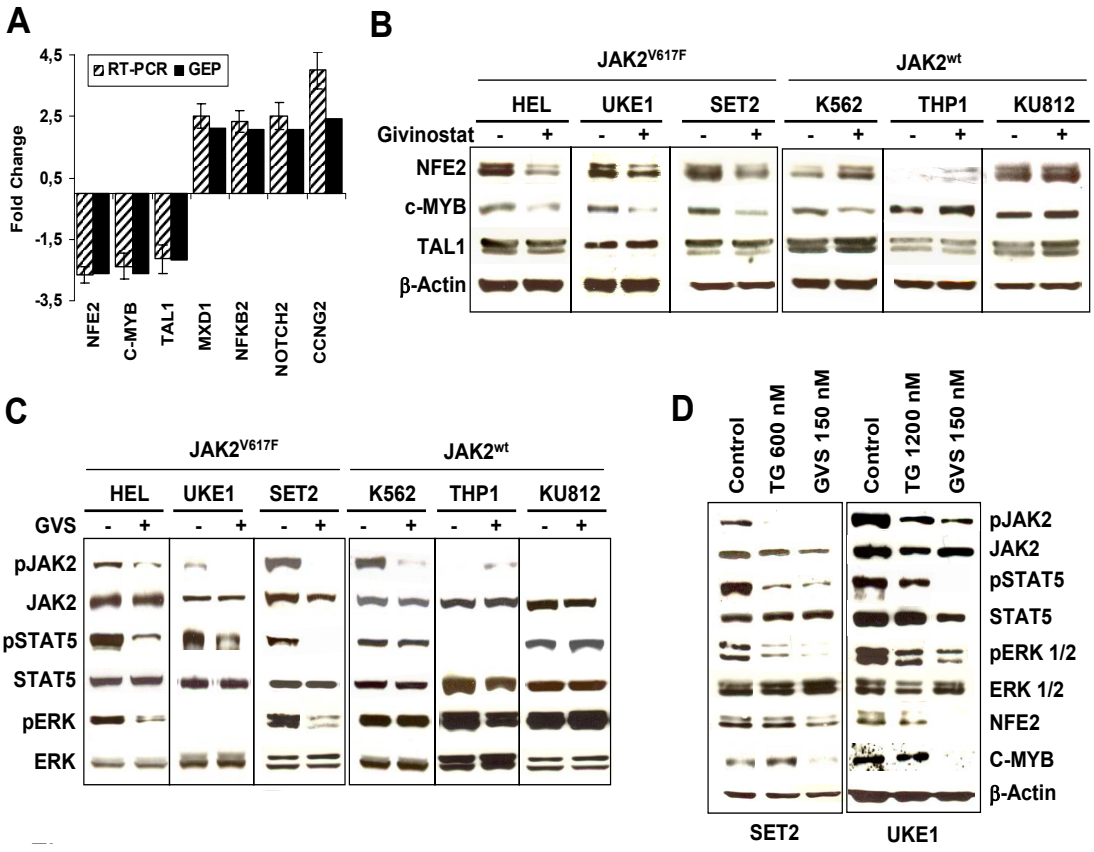


Figure 5. Effect of GVS and TG101209 on hematopoietic gene and protein expression. (A) The HEL cell line was treated with 250 nM GVS for 6 hours and RNA extracted. The expression of 7 genes was measured by RTQ-PCR (striped bars) and compared with GEP analysis (black bars). The RTQ-PCR data are the mean fold increase and standard deviations from three independent experiments. (B) Effect of 250 nM GVS (150 nM in the case of UKE1) for 24 hours on expression of the indicated proteins in JAK2V617F and JAK2wt cell lines. (C) Effect of 250 nM GVS for 24 hours (150 nM in the case of UKE1) on JAK2-STAT5 signaling in JAK2V617F and JAKwt cell lines. (D) Effect of 600 nM or 1200 nM JAK2 inhibitor TG101209 or 150 nM GVS on protein expression in SET2 or UKE1 cells. The Western blot data are representative of three independent experiments.

GVS modulates histone H3 acetylation on the NF-E2 proximal and distal promoters.

The fact that TF gene modulation was independent from the JAK2-STAT5 pathway suggested that GVS modulates these genes through direct effects on histones associated with their promoters. In order to verify this hypothesis, we performed ChIP analyses. We concentrated on the NF-E2 gene, which has two promoters called distal and proximal and placed at about _350 and _200 nucleotides from start sites, respectively. UKE1 and SET2 cells were treated for 6 hours with 250 nM GVS and ChIP performed with anti-H3, anti-H3K9ac, and anti–H3K14ac antibodies. The data demonstrate an enrichment of H3K9ac, especially on the proximal but also on the distal NF-E2 promoters in both cell lines (Fig. 6A and D). In contrast, H3K14ac was decreased after GVS treatment, although this was not significant in either cell line (Fig. 6B and E). Total H3 was not modified (Fig. 6C and F). The effect was specific for NF-E2 because it was not consistently observed in the AGTR1 control gene (Fig. 6A_F),

chosen because it is not modulated by GVS in the GEP analysis (fold change 0.93 and 1.03 in HEL and UKE1, respectively, data not shown).

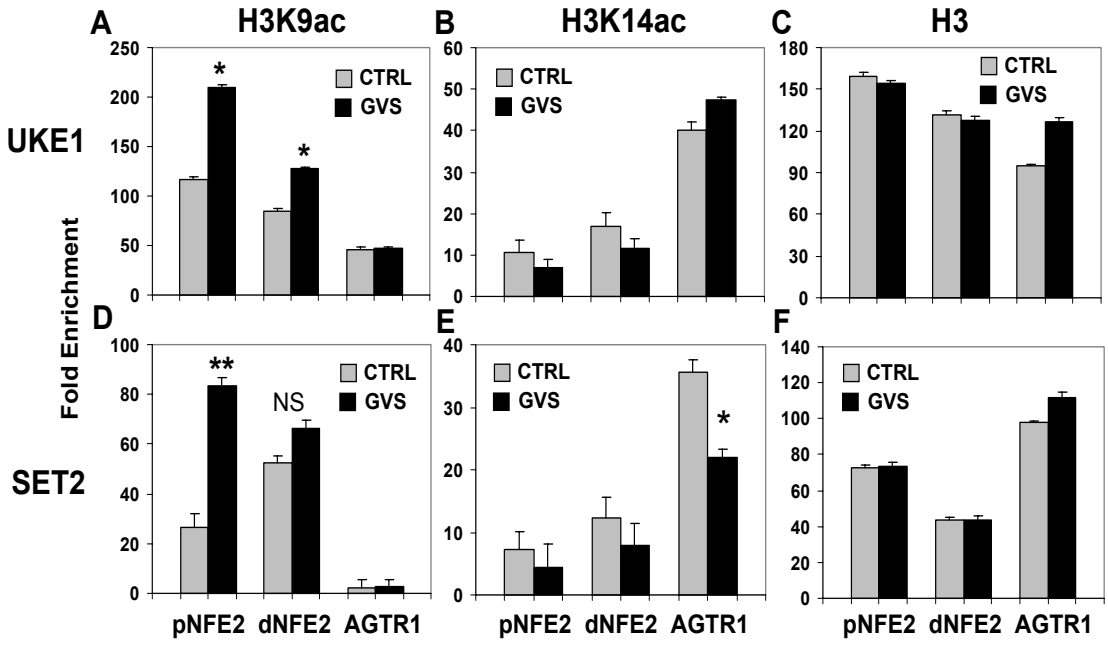


Figure 6. Enrichment of NF-E2 promoter-associated H3K9ac induced by GVS treatment. UKE1 (ALC) and SET2 (DLF) cell lines were untreated (gray bars) or treated with 250 nM GVS (black bars) for 6 hours and subjected to ChIP using H3K9ac (A, D), H3K14ac (B, E) or H3-specific antibodies (C, F). Quantitative PCR amplification of proximal (pNF-E2) and distal NF-E2 (dNF-E2) as well as control AGRT1 promoters was then performed and normalized against satellite centromeric sequences. Data are the means and standard deviations of three independent experiments, each performed in duplicate. **$p < 0.01$; *$p < 0.05$.

GVS modulates TF in MPN patients cells in vitro and inhibits erythroid differentiation .

We next determined whether TF modulation by GVS could also be observed in purified CD34+ cells from JAK2V617F MPN patients. The patient characteristics are listed in Supplementary Table E5 (online only, available at www. exphem.org). In four cases (patients 1–4), the CD34+ cells were directly treated with GVS for 24 hours and proteins extracted. In the other four cases (patients 5–8), the number of CD34+ cells recovered was too small and these were first expanded in interleukin-3 and SCF containing medium for 1 week before treatment with GVS. In all eight patient samples analyzed we found that GVS strongly inhibited NF-E2 expression (Fig. 7A). In contrast, we could not detect C-MYB in any conditions (data not shown). This was probably due in part to the difficulty of purifying CD34+ cells from MPN patients and very low amounts of protein obtained in some cases (10 mg). The data show that modulation of NF-E2 in all patients samples was similar to that observed in JAK2V617F cell lines.

Because downmodulation of NF-E2 by GVS was expected to inhibit proliferation as well as erythroid differentiation, purified CD34+ from JAK2V617F MPN patients were cultured in vitro with interleukin-3, SCF, and EPO in presence or absence of GVS. Biologically active but relatively low dose of drug was used (50 nM) in order to avoid massive induction of apoptosis. As shown in Figure 7B, CD34+ cells proliferated in presence of growth factors and GVS significantly inhibited cell growth in these conditions, with 23% and 44.4% decrease in cell number at day 3 and 5, respectively (Fig. 7B).

Upon culture for 1 week, cells differentiated toward the erythroid lineage, with a mean of 43% and 54% CD36+/CD235+ cells observed after 5 and 7 days culture, respectively. GVS significantly retarded differentiation to more mature double-positive erythroblasts with 15% to 16% reduction in CD36+/CD235+ cells present at days 5 to 7 (Fig. 7C) and corresponding increase in double-negative cells (data not shown).

We conclude that GVS downmodulates NF-E2 and inhibits proliferation and erythroid differentiation in CD34+ cells isolated from MPN patients.

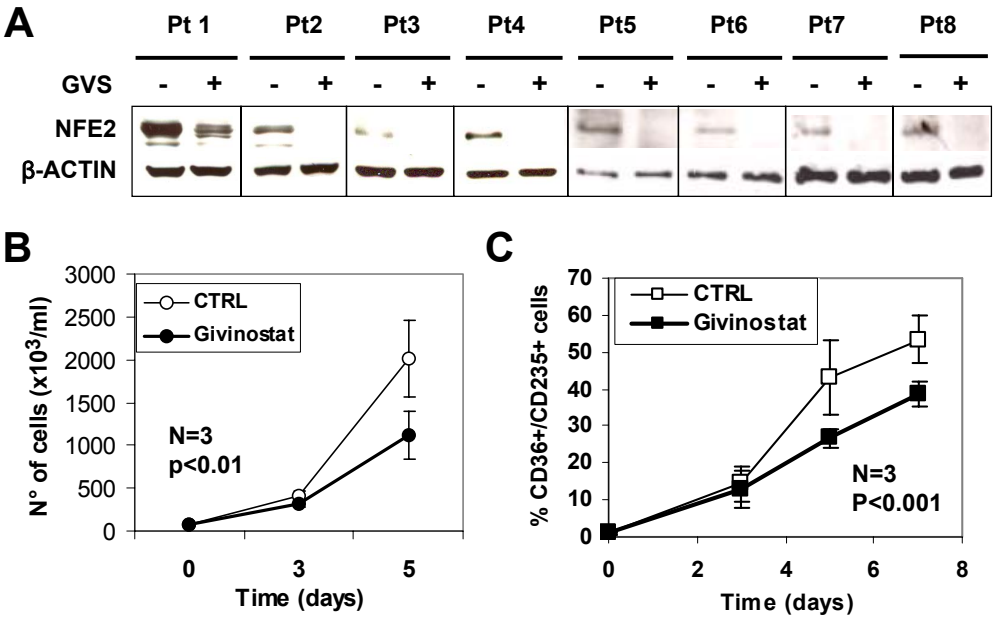


Figure 7. Effects of GVS on cells isolated from JAK2V617F MPN patients. (A) CD34+ cells were purified from eight JAK2V617F MPN patients peripheral blood. In four cases (patients 1–4), the CD34+ cells were treated immediately with 250 nM GVS for 24 hours, in the other four cases (patients

5–8) cell were first expanded in interleukin-3 and SCF for 1 week before treatment with 250 nM GVS for 24 hours. Cellular extracts were analyzed for expression of the indicated proteins. (B, C) CD34+ cells from JAK2V617F MPN patients were cultured in IL-3, SCF, and EPO and in presence or absence of 50 nM GVS and absolute live cell numbers counted at days 3 and 5 (B). Cells were also collected at day 3, 5, and 7 and differentiation measured as percentage CD36+/ CD235+ cells by flow cytometry (C). Results are the means and standard deviations of three independent experiments.

Discussion

In this report, we have investigated the biological effects and mechanism of action of GVS in MPN cells. We show that JAK2V617F cells are more sensitive to GVS by two- to threefold in terms of both inhibition of proliferation and apoptosis induction, using a panel of JAK2-mutated and wt cell lines, extending our previous observations with the sole HEL [9,10].

We next investigated the mechanism of action of GVS in JAK2V617F cells. GEP revealed 293 genes commonly modulated by GVS at 6 hours in the HEL and UKE1 cell lines. Functional analysis of the modulated genes revealed that 19 genes were related to cell cycle control. Interestingly, RGS2, CCNG2, and MLF1 were similarly found induced in a MM cell line treated with GVS [26]. Of major interest is MLF1 because this putative oncogene is fused to NPM1 in t(3;5)(q25.1;q34) bearing myelodysplastic syndrome and acute myelogenous leukemia cells. MLF1 is reported to induce p53-dependent cell cycle arrest. In addition, forced expression of MLF1 in JE2 cells inhibits erythroid and promotes monocytic differentiation [27]. MLF1 induction in JAK2-mutated cells can contribute to the cell cycle and differentiation block.

Interestingly, 10 GVS-modulated genes (i.e., APAF1, SAMHD1, TPMT, CPNE3, ANGPT1, MYCN, NF-E2, selectin P ligand, TAL1, and WT1) have been reported by other groups to be abnormally expressed in MPN patients cells [14,15,25,28]. More precisely, in 9 of 10 cases, genes deregulated in MPN patients cells with respect to controls were normalized by the drug (i.e., overexpressed genes were downmodulated by GVS, and

underexpressed genes were induced). The only exception was CPNE3, which was overexpressed in MPN compared to normal granulocytes [13] and induced by GVS in HEL/UKE1.

In addition, we found that 33 GVS-modulated genes are associated with hematopoiesis. Of these, 9 were linked to chemotaxis and adhesion and 24 were implicated in proliferation, differentiation, lineage commitment, and/or apoptosis of hematopoietic cells. Of most interest were NF-E2, C-MYB, and TAL1, all TF known to control differentiation along the erythro-megakaryocytic-myeloid lineages. We could confirm by RTQ-PCR that all three genes were modulated at the RNA level by GVS. At the protein level, we could demonstrate significant downmodulation of C-MYB and NF-E2 in all three JAK2-mutated cell lines, confirming the messenger RNA data. In contrast, we observed only a marginal downmodulation of TAL1 protein level 24 hours after GVS treatment in the same cell lines. This suggests that the TAL1 messenger RNA decrease after GVS treatment might not be sufficient or protein stability too high to lead to significantly decreased TAL1 protein expression at 24 hours.

Protein analyses of the JAK2V617F cell lines showed that GVS in all cases inhibited JAK2 and downstream STAT5 and ERK1/2 phosphorylation, confirming and extending our previously published data on HEL [10]. In contrast, GVS did not fully inhibit the JAK2-STAT5-ERK1/2 pathway in JAK2wt cells. JAK2 phosphorylation was inhibited only in K562 and not in other wt cell lines, but in all cases downstream STAT5/ERK1/2 were not affected by the drug. The results on JAK2 phosphorylation in K562 shown here are somewhat different from our previous report [10], presumably due to the different method used, i.e., extraction and quantification of total

proteins performed here, rather than extraction from fixed amount of cells without protein quantification, as done previously. The confirmed lack of phospho-STAT5/ERK1/2 inhibition in JAK2wt leukemic cells is probably due to JAK2- independent mechanisms of STAT5/ERK1/2 activation present in these cells. Indeed bcr-abl is known to activate STAT5 phosphorylation directly [29]. We suggest that inhibition of the JAK2-STAT5-ERK1/2 pathway by GVS in JAK2V617F cells is in part responsible for growth inhibition and apoptosis induction induced by GVS in these cells.

GVS, however, showed additional properties because it modulated expression of several TF, in particular NF-E2 and C-MYB independently from the JAK2-STAT5-ERK1/2 pathway, as shown by using the selective JAK2 inhibitor TG101209. Modulation of the NF-E2 and C-MYB proteins by the HDACi, therefore, presumably takes place through modification of histones associated with these gene promoters. Analysis of acetylated histone H3 by ChIP assay of the NF-E2 promoter showed that H3K9ac on both the proximal and distal promoters of NF-E2 was highly enriched in GVS-treated cells compared with controls. In contrast, H3K14ac was decreased by HDACi treatment, although not in a statistically significant manner.

The effects were specific and not observed in a control gene AGTR1, the expression of which was not modulated by GVS in GEP analysis. These data demonstrate that changes in acetylation state of specific lysine residues of histone H3 take place on the promoters of NF-E2 in response to GVS in parallel with a decreased in its transcription, suggesting a direct effect of GVS on the promoter of this gene, leading to altered expression. The vast majority of studies in which increased H3 acetylation was assessed after treatment with

HDACi, including GVS, have used antibodies directed against H3K9 and H3K14. The use of antibodies against single modifications allowed us to discriminate between the two acetylations, suggesting that HDACi might have a differential effect on other histone acetylations in a context-specific way. In particular, it is intriguing that NF-E2 transcription was found here to be repressed, with a sole effect on H3K9; examples of genes repressed by these modifications exist and gene transcription is regulated by extremely complex and still poorly understood patterns of other histones post-translational modifications than the one that could be studied here [30,31]. More work lies ahead to determine the specificity of GVS in terms of inhibition of the plethora of other important histone acetylations.

We show here for the first time that an HDACi downmodulates the NF-E2 and C-MYB transcription factors in JAK2V617F MPN cell lines and freshly isolated patients samples. This is in agreement with inhibition of NF-E2 expression in vivo in patients treated by HDAC inhibitor [32]. TF downmodulation can be an important mechanism of action of GVS in MPN, in addition to its inhibition of JAK2 phosphorylation. NF-E2 is required for megakaryo- cyte to platelet transition and has been reported to either promote or inhibit erythroid differentiation, according to the model analyzed [33]. In addition, NF-E2 is consistently overexpressed in MPN [13–15] and has been recently suggested to contribute by itself to the MPN phenotype [32].

The drug inhibited proliferation and differentiation of MPN patients cells toward the erythroid lineage in vitro. Modulation of NF-E2 may contribute to these effects, although other genes, such as C-MYB or others, can also contribute. The C-MYB TF is required at

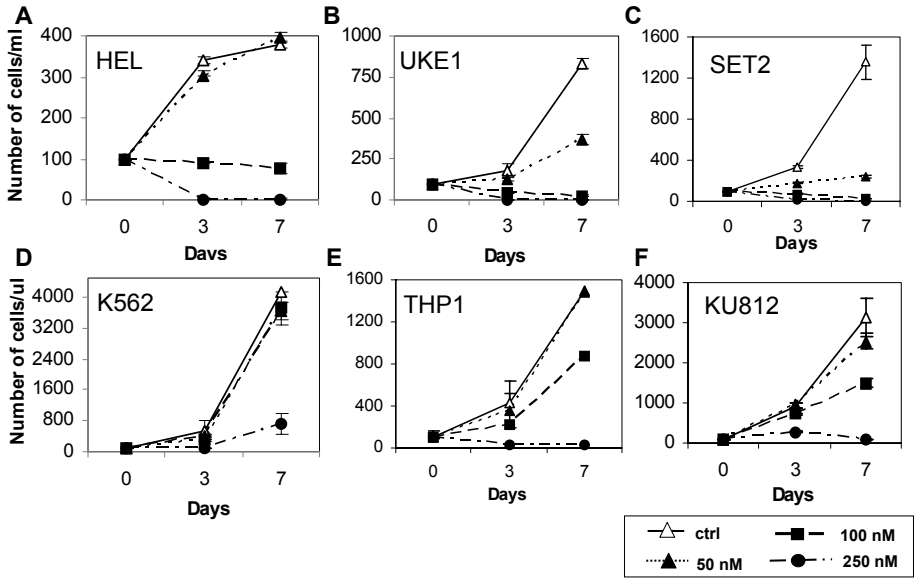
multiple stages of hematopoiesis, including long-term self-renewal of hematopoietic stem cells, fetal erythropoiesis, and commitment toward the erythroid at the expense of the monocyte and megakaryocytic lineages [34–38]. C-MYB functional mutants show a myeloproliferative-like disease [39,40]. TAL1/SCL is required for the specification of the hematopoietic lineage [41–43] and plays a role in stress thrombopoieisis and erythropoiesis [44,45]. The TAL1/GATA1/ LMO2 protein complex recognizes many elements in erythroid gene promoters. Interestingly, GEP data showed that LMO2 and GATA1 messenger RNAs were also down- modulated by GVS by 30% to 45%, i.e., at levels below the twofold cutoff value.

Although we could not demonstrate significant TAL1 protein inhibition after 24-hour drug treatment, we cannot exclude that downmodulation of TAL1/ GATA1/LMO2 messenger RNA expression contribute to the drug effects in vitro. Finally, several of the TF analyzed here, such as C-MYB and TAL1, have been reported to interact with each other [46,47], making their coordinated modulation by GVS of particular interest.

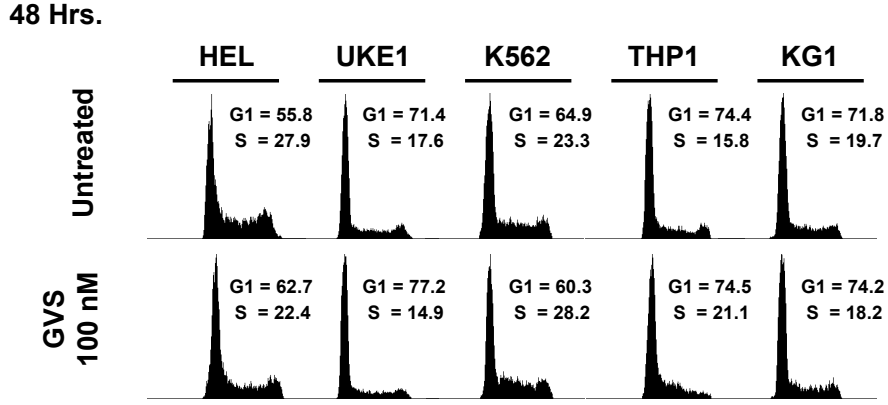
Conclusions

GVS inhibits the JAK2/STAT5/ERK1/2 pathway and independently expression of crucial hematopoietic TF, most importantly NF-E2. These effects offer an explanation for the response of JAK2V617F cells to the drug, making this compound of particular interest in the context of MPN treatment.

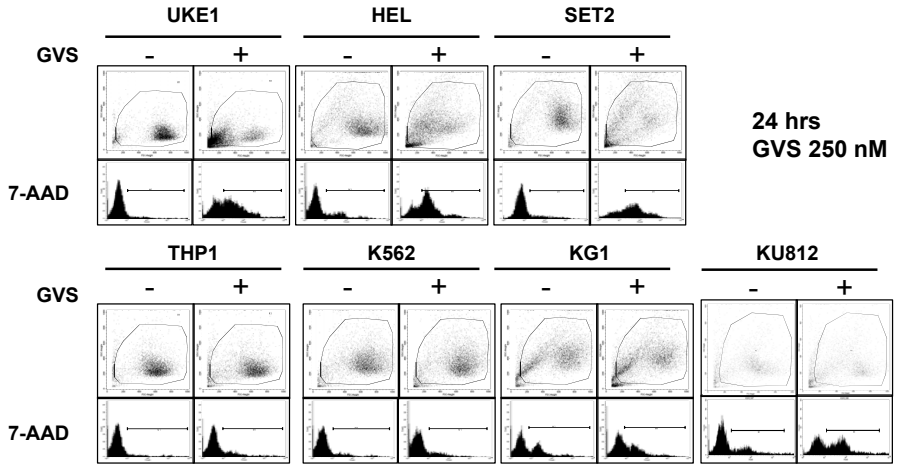
Supplementary Figure 1: Growth curves of indicated cell lines treated with 50, 100 or 250 nM GVS



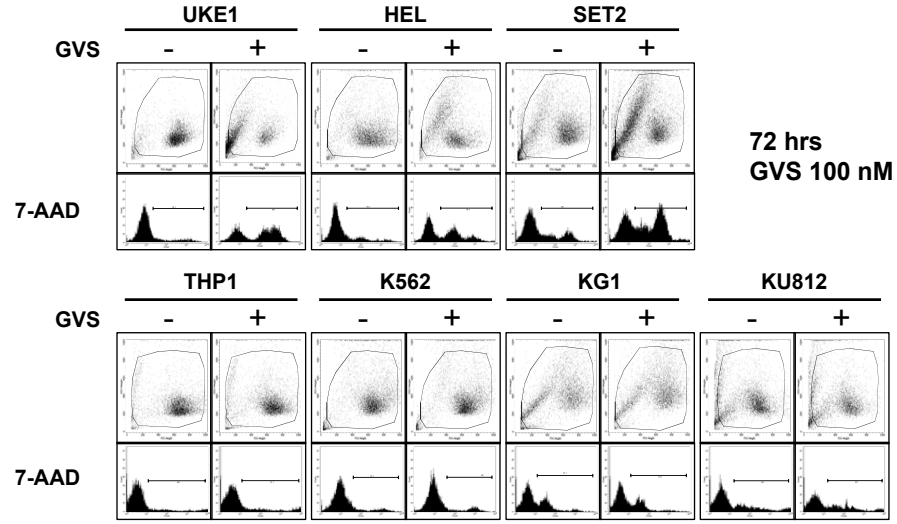
Supplementary Figure 2: Cell Cycle analysis of GVS treated cell lines. The indicated cell lines were treated with 100 nM of GVS for 48 hours and cell cycle analysis of propidium iodide stained cells performed. The data are representative of 3 independent experiments.



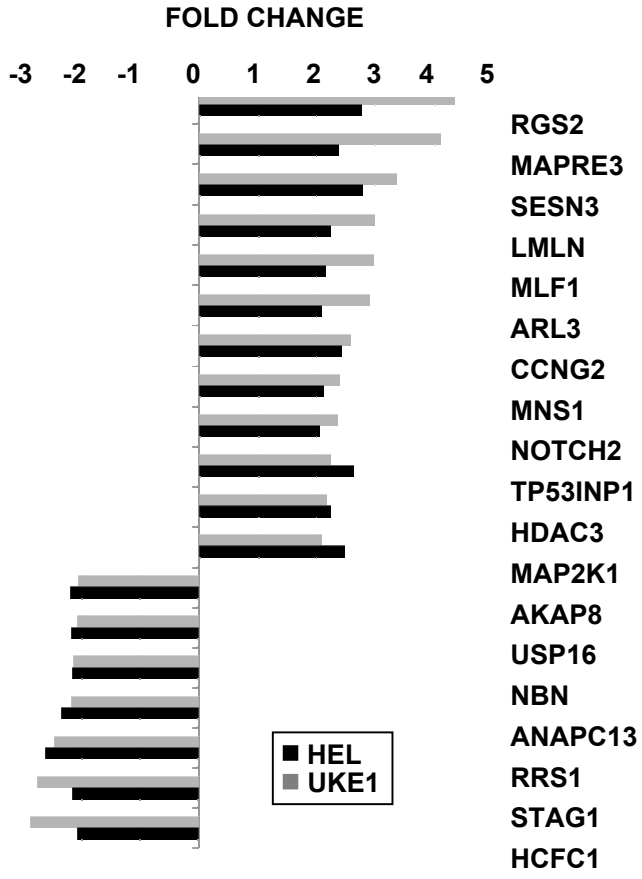
Supplementary Figure 3A: Apoptosis assays at 24 hours. The indicated JAK2^{V617F} (upper panels) and JAK2wt (lower panels) cell lines were treated for 24 hours with 250 nM GVS and stained with 7AAD. Dot plots of forward and side scatters and 7AAD fluorescence histograms are shown. Results are representative of 3 independent experiments.



Supplementary Figure 3B: Apoptosis assays at 72 hours. The indicated JAK2^{V617F} (upper panels) and JAKwt (lower panels) cell lines were treated for 72 hours with 100 nM GVS and stained with 7AAD. Dot plots of forward and side scatters and 7AAD fluorescence histograms are shown. Results are representative of 3 independent experiments.



Supplementary Figure 4. : Cell cycle associated genes modulated by GVS in HEL and UKE1. The 19 cell cycle associated genes modulated by GVS in both HEL (black bars) and UKE1 (grey bars) are shown.



Supplementary Table 1. List of primers used for quantitative PCR	
Primers	**Sequences**
1.Primers for RTQ-PCR:	
RPL13A forward	5'-CCTGGAGGAGAAGAGGAAAGAGA-3'
RPL13A reverse	5'-TTGAGGACCTCTGTGTATTTGTCAA-3'
NFE2 forward	5'-TGACTTGGCAGGAGATCATGTC-3'
NFE2 reverse	5'-GAGGAAGTGGGAAGCCAGAATC-3'
C-MYB forward	5'-GTGGCATAACCACTTGAATCCAG-3'
C-MYB reverse	5'-TAGCATTATCAGTTCGTCCAGGC-3'
TAL1 forward	5'-GAGGGGTTGTTGTTGCTGTTG-3'
TAL1 reverse	5'-CAGGTGTTTGGAGCCTTTCC-3'
NFKB2 forward	5'-GAGAACGGAGACACACCACTGC-3'
NFKB2 reverse	5'-TGGTTGGTGAGGTTGACAACG-3'
NOTCH2 forward	5'-GCCTCCAGGAGAGGTGTGCT-3'
NOTCH2 reverse	5'-CAGGGGGCACTGACAGTAATG-3'
MXD1 forward	5'-GGGAGAGAGAAGCTGAACATGGT-3'
MXD1 reverse	5'-AGCCCGTCTATTCTTCTCCATTTC-3'
CCNG2 forward	5'-ATGAAGGGGTCCAACTTCTCG-3'
CCNG2 reverse	5'-GTGTTATCATTCTCCGGGGTAGC-3'
2. Primers for ChIP assays:	
dNFE2 promoter forward	5'-GAGAGGCTTGGAGCCATGT-3'
dNFE2 promoter reverse	5'- GTGGCTCTCCTCTCCTCCTG-3'
pNFE2 promoter forward	5'- GGGTTAAGGTATGGCCCAAAT-3'
pNFE2 promoter reverse	5'-CCTGGGCCAGATAAGAGGTT-3'
AGTR1 promoter forward	5'-GTATCCCTTGCACAGCATCC-3'
AGTR1 promoter reverse	5'-TTGCTCTACCAGGAGCAGGT-3'
SAT_CEN forward	5'-GGCGACCAATAGCCAAAAAGTGAG-3'
SAT:CEN reverse	5'-CAATTATCCCTTCGGGGAATCGG-3'

Supplemetary Table 2:

Gene Symbol	Gene Title	HEL FC	UKE1 FC
GLRX	glutaredoxin (thioltransferase)	6,53	2,33
STAT4	signal transducer and activator of transcription 4	6,29	4,52
ELOVL4	elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 4	6,14	2,71
C7orf23	chromosome 7 open reading frame 23	5,42	2,82
TMOD2	tropomodulin 2 (neuronal)	4,51	5,56
SGTB	small glutamine-rich tetratricopeptide repeat (TPR) containing, beta	4,12	2,46
MPZL1	myelin protein zero-like 1	4,04	4,89
FAM49A	family with sequence similarity 49, member A	3,94	3,97
TTLL7	tubulin tyrosine ligase-like family, member 7	3,81	2,26
RGL1	ral guanine nucleotide dissociation stimulator-like 1	3,76	5,71
ROPN1L	ropporin 1-like	3,74	4,22
CTTNBP2NL	CTTNBP2 N-terminal like	3,65	3,01
SORT1	sortilin 1	3,63	2,29
MEGF9	multiple EGF-like-domains 9	3,58	4,16
DIXDC1	DIX domain containing 1	3,53	2,44
PAG1	phosphoprotein associated with glycosphingolipid microdomains 1	3,51	2,97
PCYOX1	prenylcysteine oxidase 1	3,51	3,17
ASMTL	acetylserotonin O-methyltransferase-like	3,44	2,49
DHRS1	dehydrogenase/reductase (SDR family) member 1	3,43	2,51
CYB5R1	cytochrome b5 reductase 1	3,36	2,46
VAMP1	vesicle-associated membrane protein 1 (synaptobrevin 1)	3,20	3,78
MYBL1	v-myb myeloblastosis viral oncogene homolog (avian)-like 1	3,20	7,34
FUCA1	fucosidase, alpha-L- 1, tissue	3,19	2,78
CCDC112	coiled-coil domain containing 112	3,18	2,41
SLC31A1	solute carrier family 31 (copper transporters), member 1	3,16	2,72
RAB8B	RAB8B, member RAS oncogene family	3,14	2,12
PGM2L1	phosphoglucomutase 2-like 1	3,12	2,06
LOC387790	hypothetical LOC387790	3,11	2,71
SAMHD1	SAM domain and HD domain 1	3,10	5,73
ARMC9	armadillo repeat containing 9	3,09	3,47
FBXL2	F-box and leucine-rich repeat protein 2	3,04	2,17
AKTIP	AKT interacting protein	3,04	2,49
RBM11	RNA binding motif protein 11	3,03	2,99
CPNE3	copine III	2,99	2,03
EFNA4	ephrin-A4	2,96	3,32
ITSN1	intersectin 1 (SH3 domain protein)	2,95	2,55
APAF1	apoptotic peptidase activating factor 1	2,91	2,26
GPR155	G protein-coupled receptor 155	2,88	2,63
TPMT	thiopurine S-methyltransferase	2,88	2,66
STK17B	serine/threonine kinase 17b	2,86	2,48
SEC61A2	Sec61 alpha 2 subunit (S. cerevisiae)	2,86	2,36
TRIM26	tripartite motif-containing 26	2,84	2,23
OAZ2	ornithine decarboxylase antizyme 2	2,83	2,03
SCML1	sex comb on midleg-like 1 (Drosophila)	2,83	4,00

C20orf74	chromosome 20 open reading frame 74	2,82	2,07
SESN3	sestrin 3	2,79	3,36
SERPINI1	serpin peptidase inhibitor, clade I (neuroserpin), member 1	2,78	7,22
RGS2	regulator of G-protein signaling 2, 24kDa	2,77	4,35
RASGEF1B	RasGEF domain family, member 1B	2,76	2,17
MCTP1	multiple C2 domains, transmembrane 1	2,73	2,27
EXTL2	exostoses (multiple)-like 2	2,72	2,27
PLS1	plastin 1 (I isoform)	2,72	2,18
TSPAN13	tetraspanin 13	2,72	3,38
RNF122	ring finger protein 122	2,70	2,17
SERAC1	serine active site containing 1	2,67	2,60
CREB3	cAMP responsive element binding protein 3	2,65	2,44
C4orf33	chromosome 4 open reading frame 33	2,64	2,12
POLB	polymerase (DNA directed), beta	2,64	2,26
CCDC28A	coiled-coil domain containing 28A	2,63	2,75
TRIM38	tripartite motif-containing 38	2,63	2,11
PELI1	pellino homolog 1 (Drosophila)	2,62	2,19
TP53INP1	tumor protein p53 inducible nuclear protein 1	2,62	2,22
VCL	vinculin	2,61	2,07
CCDC148	coiled-coil domain containing 148	2,61	5,40
PAIP2B	poly(A) binding protein interacting protein 2B	2,60	3,91
FAM45A	family with sequence similarity 45, member A	2,60	2,05
BBS12	Bardet-Biedl syndrome 12	2,60	2,93
YPEL2	yippee-like 2 (Drosophila)	2,58	2,36
HDAC11	histone deacetylase 11	2,56	2,19
TEX9	testis expressed 9	2,56	2,30
SMOX	spermine oxidase	2,56	2,23
FBXO48	F-box protein 48	2,56	3,10
FAM154B	family with sequence similarity 154, member B	2,55	2,86
FGD6	FYVE, RhoGEF and PH domain containing 6	2,52	3,00
GBP5	guanylate binding protein 5	2,52	2,07
PLA2G4A	phospholipase A2, group IVA (cytosolic, calcium-dependent)	2,51	2,14
TMEM55A	transmembrane protein 55A	2,51	2,02
KDELC1	KDEL (Lys-Asp-Glu-Leu) containing 1	2,50	2,88
RWDD2A	RWD domain containing 2A	2,49	2,86
MAP2K1	mitogen-activated protein kinase kinase 1	2,48	2,10
STK38L	serine/threonine kinase 38 like	2,46	2,42
C1orf38	chromosome 1 open reading frame 38	2,46	2,36
CX3CR1	chemokine (C-X3-C motif) receptor 1	2,45	2,15
ZSWIM6	zinc finger, SWIM-type containing 6	2,45	2,44
FAM126A	family with sequence similarity 126, member A	2,45	2,73
TMEM63C	transmembrane protein 63C	2,43	2,26
CCNG2	cyclin G2	2,41	2,58
MAP3K13	mitogen-activated protein kinase kinase kinase 13	2,41	2,53
TXK	TXK tyrosine kinase	2,40	2,22
TWSG1	twisted gastrulation homolog 1 (Drosophila)	2,40	2,32
GLCE	glucuronic acid epimerase	2,40	2,32
HEG1	HEG homolog 1 (zebrafish)	2,40	2,69
FAM164A	family with sequence similarity 164, member A	2,40	2,09
TMEM205	transmembrane protein 205	2,39	2,14

PLA2G15	phospholipase A2, group XV	2,38	2,52
CALCOCO1	calcium binding and coiled-coil domain 1	2,37	2,03
ANKRD13A	ankyrin repeat domain 13A	2,37	2,21
F2RL1	coagulation factor II (thrombin) receptor-like 1	2,37	2,46
TPST1	tyrosylprotein sulfotransferase 1	2,37	3,10
SGPL1	sphingosine-1-phosphate lyase 1	2,36	2,13
PURG	purine-rich element binding protein G	2,36	2,16
MAPRE3	microtubule-associated protein, RP/EB family, member 3	2,36	4,11
KDM5B	lysine (K)-specific demethylase 5B	2,35	2,19
CDC42EP3	CDC42 effector protein (Rho GTPase binding) 3	2,35	3,41
HIST3H2BB	histone cluster 3, H2bb	2,34	3,55
ACADSB	acyl-Coenzyme A dehydrogenase, short/branched chain	2,33	2,04
ABCB9	ATP-binding cassette, sub-family B (MDR/TAP), member 9	2,32	2,37
RAB43	RAB43, member RAS oncogene family	2,32	2,11
FAM107B	family with sequence similarity 107, member B	2,31	2,74
GSDMB	gasdermin B	2,30	3,88
GRK5	G protein-coupled receptor kinase 5	2,30	2,11
ENO2	enolase 2 (gamma, neuronal)	2,30	2,72
TESK2	testis-specific kinase 2	2,28	3,26
MAP1B	microtubule-associated protein 1B	2,28	2,14
HIBCH	3-hydroxyisobutyryl-Coenzyme A hydrolase	2,27	2,19
EXT1	exostoses (multiple) 1	2,26	2,03
SH3PXD2B	SH3 and PX domains 2B	2,25	2,10
HIST2H4A	histone cluster 2, H4a	2,25	5,79
HDAC3	histone deacetylase 3	2,24	2,16
LMLN	leishmanolysin-like (metallopeptidase M8 family)	2,24	3,00
PTPN13	protein tyrosine phosphatase, non-receptor type 13 (APO-1/CD95 (Fas)-associated phosphatase)	2,21	2,95
ATL1	atlastin GTPase 1	2,21	2,36
INPP5K	inositol polyphosphate-5-phosphatase K	2,21	2,52
WDR19	WD repeat domain 19	2,20	3,71
ZSCAN16	zinc finger and SCAN domain containing 16	2,20	2,56
IDH1	isocitrate dehydrogenase 1 (NADP+), soluble	2,19	2,06
ATG2A	ATG2 autophagy related 2 homolog A (S. cerevisiae)	2,19	2,08
H1F0	H1 histone family, member 0	2,18	4,43
SLFN5	schlafen family member 5	2,18	4,01
ACER2	alkaline ceramidase 2	2,17	2,47
EPS8	epidermal growth factor receptor pathway substrate 8	2,15	2,35
IFT80	intraflagellar transport 80 homolog (Chlamydomonas)	2,15	2,36
ANO10	anoctamin 10	2,15	2,12
MLF1	myeloid leukemia factor 1	2,15	2,98
SBF2	SET binding factor 2	2,14	2,13
FNBP1L	formin binding protein 1-like	2,14	2,12
WDR31	WD repeat domain 31	2,13	2,22
RP1-199H16.1	similar to OTTHUMP00000028720	2,13	3,48
MNS1	meiosis-specific nuclear structural 1	2,13	2,39
MAP1A	microtubule-associated protein 1A	2,13	2,58
WDR47	WD repeat domain 47	2,13	2,36
EPAS1	endothelial PAS domain protein 1	2,12	2,21
SIDT2	SID1 transmembrane family, member 2	2,12	2,28

RAB30	RAB30, member RAS oncogene family	2,12	2,29
ATP6V1G2	ATPase, H+ transporting, lysosomal 13kDa, V1 subunit G2	2,11	3,22
SLC4A5	solute carrier family 4, sodium bicarbonate cotransporter, member 5	2,11	3,94
MXD1	MAX dimerization protein 1	2,11	2,33
KIF3A	kinesin family member 3A	2,11	4,01
NFIL3	nuclear factor, interleukin 3 regulated	2,11	3,12
BIRC3	baculoviral IAP repeat-containing 3	2,10	2,32
HPS3	Hermansky-Pudlak syndrome 3	2,10	2,02
NEU1	sialidase 1 (lysosomal sialidase)	2,10	3,66
PRKACA	protein kinase, cAMP-dependent, catalytic, alpha	2,09	2,22
ARL3	ADP-ribosylation factor-like 3	2,09	2,89
EFNA3	ephrin-A3	2,09	2,25
UNC13B	unc-13 homolog B (C. elegans)	2,09	2,75
NFKB2	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	2,09	2,60
CEP70	centrosomal protein 70kDa	2,08	2,00
PLEKHM3	pleckstrin homology domain containing, family M, member 3	2,08	2,41
GATSL3	GATS protein-like 3	2,07	2,60
SLC46A3	solute carrier family 46, member 3	2,07	3,12
KLHL24	kelch-like 24 (Drosophila)	2,06	2,87
SORBS1	sorbin and SH3 domain containing 1	2,06	2,66
ALS2	amyotrophic lateral sclerosis 2 (juvenile)	2,06	2,03
KIAA1632	KIAA1632	2,06	2,02
NOTCH2	Notch homolog 2 (Drosophila)	2,06	2,35
IFI30	interferon, gamma-inducible protein 30	2,05	2,73
ELL2	elongation factor, RNA polymerase II, 2	2,05	2,10
SGK269	NKF3 kinase family member	2,04	2,19
BCL2L11	BCL2-like 11 (apoptosis facilitator)	2,04	2,11
GABARAPL1	GABA(A) receptor-associated protein like 1	2,04	2,16
FARP2	FERM, RhoGEF and pleckstrin domain protein 2	2,03	2,15
ACO1	aconitase 1, soluble	2,03	2,01
HSDL2	hydroxysteroid dehydrogenase like 2	2,02	2,15
DNAJC28	DnaJ (Hsp40) homolog, subfamily C, member 28	2,02	2,22
PI4K2A	phosphatidylinositol 4-kinase type 2 alpha	2,02	3,96
BTN2A2	butyrophilin, subfamily 2, member A2	2,02	2,10
STAP1	signal transducing adaptor family member 1	2,01	2,05
RUNDC3A	RUN domain containing 3A	2,01	2,33
FAM20B	family with sequence similarity 20, member B	-2,00	-2,18
SELPLG	selectin P ligand	-2,00	-3,40
BRD9	bromodomain containing 9	-2,01	-2,09
GMEB1	glucocorticoid modulatory element binding protein 1	-2,03	-2,05
MRM1	mitochondrial rRNA methyltransferase 1 homolog (S. cerevisiae)	-2,05	-2,45
SFXN4	sideroflexin 4	-2,05	-2,07
PUS7	pseudouridylate synthase 7 homolog (S. cerevisiae)	-2,05	-2,18
COQ7	coenzyme Q7 homolog, ubiquinone (yeast)	-2,05	-2,43
GEMIN4	gem (nuclear organelle) associated protein 4	-2,06	-2,16
NMI	N-myc (and STAT) interactor	-2,06	-3,05
HCFC1	host cell factor C1 (VP16-accessory protein)	-2,06	-2,86
CCDC26	coiled-coil domain containing 26	-2,08	-4,54

MCM9	minichromosome maintenance complex component 9	-2,08	-2,62
GTF3C6	general transcription factor IIIC, polypeptide 6, alpha 35kDa	-2,10	-2,10
GABPB1	GA binding protein transcription factor, beta subunit 1	-2,10	-2,10
THOC6	THO complex 6 homolog (Drosophila)	-2,10	-2,17
SNORA62	small nucleolar RNA, H/ACA box 62	-2,10	-2,22
DTX3L	deltex 3-like (Drosophila)	-2,10	-2,86
PSTK	phosphoseryl-tRNA kinase	-2,12	-2,02
SMYD5	SMYD family member 5	-2,12	-2,16
TREML2	triggering receptor expressed on myeloid cells-like 2	-2,12	-2,21
DUS3L	dihydrouridine synthase 3-like (S. cerevisiae)	-2,13	-2,57
NBN	nibrin	-2,13	-2,12
STAG1	stromal antigen 1	-2,13	-2,75
NETO2	neuropilin (NRP) and tolloid (TLL)-like 2	-2,13	-2,95
SNORA3	small nucleolar RNA, H/ACA box 3	-2,14	-2,70
RAD9B	RAD9 homolog B (S. pombe)	-2,14	-2,12
WT1	Wilms tumor 1	-2,14	-2,01
UNQ3104	ACAH3104	-2,14	-2,72
YARS2	tyrosyl-tRNA synthetase 2, mitochondrial	-2,14	-2,25
SNORA27	small nucleolar RNA, H/ACA box 27	-2,14	-2,21
GTF2I	general transcription factor IIi	-2,15	-2,85
USP16	ubiquitin specific peptidase 16	-2,16	-2,05
AKAP8	A kinase (PRKA) anchor protein 8	-2,17	-2,04
TAL1	T-cell acute lymphocytic leukemia 1	-2,17	-2,42
ABCE1	ATP-binding cassette, sub-family E (OABP), member 1	-2,18	-2,01
SNORA24	small nucleolar RNA, H/ACA box 24	-2,18	-2,58
OTUD4	OTU domain containing 4	-2,19	-2,02
CTSO	cathepsin O	-2,21	-2,22
METTL1	methyltransferase like 1	-2,22	-2,25
RASGRP3	RAS guanyl releasing protein 3 (calcium and DAG-regulated)	-2,22	-2,62
B3GNT5	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 5	-2,23	-2,53
SLC24A3	solute carrier family 24 (sodium/potassium/calcium exchanger), member 3	-2,26	-2,05
RABL3	RAB, member of RAS oncogene family-like 3	-2,26	-2,06
APOB48R	apolipoprotein B48 receptor	-2,26	-2,80
CD244	CD244 molecule, natural killer cell receptor 2B4	-2,27	-2,44
NIPAL2	NIPA-like domain containing 2	-2,27	-2,83
MPDU1	mannose-P-dolichol utilization defect 1	-2,27	-2,29
UBXN8	UBX domain protein 8	-2,27	-2,13
ZFP91-CNTF	ZFP91-CNTF readthrough transcript	-2,28	-2,46
PDCL3	phosducin-like 3	-2,30	-2,51
CAD	carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase	-2,31	-2,40
ZNF692	zinc finger protein 692	-2,31	-2,62
CTCF	CCCTC-binding factor (zinc finger protein)	-2,32	-2,14
ANAPC13	anaphase promoting complex subunit 13	-2,33	-2,15
C1orf163	chromosome 1 open reading frame 163	-2,34	-2,44
N6AMT2	N-6 adenine-specific DNA methyltransferase 2 (putative)	-2,35	-2,46
C14orf102	chromosome 14 open reading frame 102	-2,37	-2,98
TTC27	tetratricopeptide repeat domain 27	-2,37	-2,56

KAT5	K(lysine) acetyltransferase 5	-2,39	-2,32
SLC37A1	solute carrier family 37 (glycerol-3-phosphate transporter), member 1	-2,40	-2,64
U2AF2	U2 small nuclear RNA auxiliary factor 2	-2,42	-2,47
NOP16	NOP16 nucleolar protein homolog (yeast)	-2,42	-2,36
LARP7	La ribonucleoprotein domain family, member 7	-2,45	-2,34
PRDM10	PR domain containing 10	-2,46	-2,82
MRTO4	mRNA turnover 4 homolog (S. cerevisiae)	-2,51	-2,68
PTPN7	protein tyrosine phosphatase, non-receptor type 7	-2,51	-6,07
MMP14	matrix metallopeptidase 14 (membrane-inserted)	-2,51	-2,13
APBB1IP	amyloid beta (A4) precursor protein-binding, family B, member 1 interacting protein	-2,51	-2,04
FDXACB1	ferredoxin-fold anticodon binding domain containing 1	-2,52	-2,14
NOL6	nucleolar protein family 6 (RNA-associated)	-2,54	-2,24
C1orf107	chromosome 1 open reading frame 107	-2,56	-2,08
ARMC6	armadillo repeat containing 6	-2,57	-2,79
RRS1	RRS1 ribosome biogenesis regulator homolog (S. cerevisiae)	-2,60	-2,46
TRIM8	tripartite motif-containing 8	-2,61	-4,31
NFE2	nuclear factor (erythroid-derived 2), 45kDa	-2,61	-2,93
CXCL2	chemokine (C-X-C motif) ligand 2	-2,63	-2,02
SNORA45	small nucleolar RNA, H/ACA box 45	-2,64	-2,53
ANGPT1	angiopoietin 1	-2,64	-4,87
MYB	v-myb myeloblastosis viral oncogene homolog (avian)	-2,65	-2,58
AVEN	apoptosis, caspase activation inhibitor	-2,65	-2,83
ZFAT	zinc finger and AT hook domain containing	-2,66	-2,75
WDR4	WD repeat domain 4	-2,68	-2,53
TOE1	target of EGR1, member 1 (nuclear)	-2,68	-2,81
MAP1D	methionine aminopeptidase 1D	-2,70	-3,00
SPRYD4	SPRY domain containing 4	-2,71	-2,21
SLFN14	schlafen family member 14	-2,72	-2,50
GTF2IRD2	GTF2I repeat domain containing 2	-2,77	-2,64
SNORA13	small nucleolar RNA, H/ACA box 13	-2,80	-2,96
CTPS	CTP synthase	-2,80	-3,01
MEPCE	methylphosphate capping enzyme	-2,81	-2,49
MYCN	v-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian)	-2,83	-2,23
EMG1	EMG1 nucleolar protein homolog (S. cerevisiae)	-2,86	-2,30
KCTD15	potassium channel tetramerisation domain containing 15	-2,87	-2,96
GTF2IRD2B	GTF2I repeat domain containing 2B	-2,87	-2,60
ZFX	zinc finger protein, X-linked	-2,92	-4,27
RGS18	regulator of G-protein signaling 18	-2,92	-4,35
CD84	CD84 molecule	-2,96	-4,07
THADA	thyroid adenoma associated	-3,01	-2,30
CXorf26	chromosome X open reading frame 26	-3,01	-2,25
SNORA41	small nucleolar RNA, H/ACA box 41	-3,01	-2,78
SNORD49A	small nucleolar RNA, C/D box 49A	-3,06	-2,43
SNORD102	small nucleolar RNA, C/D box 102	-3,08	-2,67
EPC1	enhancer of polycomb homolog 1 (Drosophila)	-3,13	-3,38
PPRC1	peroxisome proliferator-activated receptor gamma, coactivator-related 1	-3,17	-3,15
RSAD2	radical S-adenosyl methionine domain containing 2	-3,34	-3,26

SNORD105	small nucleolar RNA, C/D box 105	-3,59	-2,66
PIGW	phosphatidylinositol glycan anchor biosynthesis, class W	-3,64	-4,04
SNORD82	small nucleolar RNA, C/D box 82	-3,75	-3,04
SNORD51	small nucleolar RNA, C/D box 51	-3,86	-3,78
SNORD20	small nucleolar RNA, C/D box 20	-3,88	-2,35
GPR85	G protein-coupled receptor 85	-4,67	-3,22
LANCL2	LanC lantibiotic synthetase component C-like 2 (bacterial)	-4,96	-4,10
UTP20	UTP20, small subunit (SSU) processome component, homolog (yeast)	-5,45	-6,21

list of the 293 commonly modulated genes in GVS versus untreated HEL and UKE1 cell lines at 6 hours. Genes are ordered according to average fold change (FC) in expression levels

Supplementary Table 3. Functional annotation clustering analysis of genes commonly modulated in HEL and UKE-1 cells by Givinostat at 6 hours.

Annotation Cluster	Representative Annotation terms	Enrichment Score*	Count
1	cell cycle	1.48	19
2	cellular amino acid and derivative metabolic process	1.42	14
3	methyltransferase activity	1.39	7

*Only annotation clusters with Enrichment score >1.3 are represented and the associated annotation terms were manually selected.

Supplementary Table 4. Characteristics of JAK2 ^{V617F} PV Patients

Patient	Gender /Age	Status JAK2^{V617F}	Hemoglobin grams	Hematocrit %	WBC 10^9/L	Platelets 10^9/L
1	M/65	Homozygous	14.0	45.7	9.1	159
2	F/75	Homozygous	13.6	45.0	18.6	510
3	M/68	Heterozygous	18.3	53.0	7.5	323
4	M/66	Heterozygous	14.0	49.8	12.0	246
5	M/42	Homozygous	16.5	51.5	8.3	249
6	F/57	Homozygous	13.5	45.7	5.9	494
7	M/68	Heterozygous	18.3	53.0	7.0	323
8	M/37	Heterozygous	15.8	48.8	11.7	893

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Chapter 3

Givinostat and Hydroxyurea synergize in vitro to induce apoptosis of cells from JAK2^{V617F} Myeloproliferative Neoplasm patients

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Abstract

We investigated whether clinically achievable concentrations of the histone deacetylase (HDAC) inhibitors givinostat and hydroxyurea induce synergistic cytotoxicity in Jak2V617F cells in vitro and through which possible mechanism. Givinostat and hydroxyurea at low doses potentiated the pro-apoptotic effects of each other in the Jak2V617F HEL and UKE1 cell lines. Givinostat induced 6.8%–20.8% and hydroxyurea (HU) 20.4%–42.4% cell death alone and 35.8%–75.3% in combination. The effect was statistically significant using the median effect Chou-Talalay method, resulting in a combination index less than 1, indicating synergy. Givinostat alone induced cell cycle arrest of the cell lines in G0/G1 and hydroxyurea in S phase, whereas both drugs together led to a G1 block. At the molecular level, hydroxyurea counteracted the induction of p21CDKN1A by Givinostat and potentiated caspase 3 activation, explaining at least in part the increased apoptosis observed in presence of both compounds. We also verified the effect of the same drugs in colony assays of freshly isolated Jak2V617F polycythemia vera cells. In this case, low doses of the compounds were additive to each other. These results suggest that combined treatment with givinostat and hydroxyurea is a potential strategy for the management of Jak2V617F myeloproliferative neoplasms.

Introduction

Philadelphia-negative myeloproliferative neoplasms (MPN) are clonal hematopoietic stem cell (HSC) diseases characterized by a variable degree of erythrocytosis, thrombocytosis and leukocytosis, as well as JAK2 mutations [1]. Mice carrying $JAK2^{V617F}$ recapitulate several features of human polycythemia vera (PV), including marked erythrocytosis and a tendency to develop myelofibrosis, confirming the pathogenic role of this mutation [2].

Although MPN are considered relatively indolent diseases, patients are at lifelong increased risk of thrombosis, hemorrhage, and myelofibrotic or leukemic transformation [3]. The therapeutic options in PV include phlebotomy, aspirin and hydroxyurea (HU, hydroxycarbamide). HU is an oral cytostatic agent that works through inhibition of ribonucleotide reductase, deoxynucleotide production and DNA synthesis. It is widely used as a first line myelosuppressive therapy for these patients [4, 5], and the control of the thrombocytosis is usually rapid and effective, even though the reduction $JAK2^{V617F}$ allele burden is still controversial [6-8]. HU also prevents ischemic attacks in PV and Essential Thrombocytemia (ET), probably through nitric oxide production and vasodilation. However HU induces several side effects, especially upon long term usage [9]. Furthermore up to 15-25% of patients show resistance or intolerance to HU. Thus combined use in MPN of HU with other agents may enable reduced doses of this drug, improved efficacy and reduced side effects. Recently JAK2 inhibitors have entered clinical trials for JAK2 mutated MPN. While these drugs have allowed improvements of systemic symptoms and splenomegaly, they do not eradicate the

malignant clone [10, 11]. In recent years histone deacetylases (HDACs) have been recognized as promising targets for MPN treatment. In particular Givinostat (ITF2357, GVS) is a hydroxamate pan-HDAC inhibitor that has been recently shown to selectively induce apoptosis of JAK2^{V617F} MPN cells. In vitro, GVS allows preferential outgrowth of JAK2 wild type over JAK2^{V617F} colonies. The drug downmodulates phosphorylated JAK2 and downstream STAT5/STAT3 [12] and therefore affects genes targeted by this pathway. It also modulates genes involved in hematopoietic differentiation through direct effects on histone modifications [13].

One of the common biological effects of HDAC inhibitors in different cellular contexts is to induce p21CDKN1A (WAF1/CIP1) expression in a p53 independent manner, therefore leading to cell cycle arrest in the G1 phase [14-17]. In vivo, the results of a Phase I clinical trial confirmed that GVS is an active drug in patients with MPN bearing JAK2^{V617F} mutation, reducing pruritus, spleen size and requirement for phlebotomy, and also reducing the JAK2^{V617F} allele burden [10].

Since HU has been reported to be synergistic with the HDAC inhibitor valproic acid in melanoma cells [18] and in view of the use of GVS and HU individually in MPN treatment, we investigated the effect of this drug combination in vitro using JAK2^{V617F} cell lines and patients cells.

Materials and Methods

Reagents and antibodies

Diffuse large Givinostat (ITF2357 Italfarmaco, Milano, Italy; patent WO97/43251, US6034096) was synthesized by Italfarmaco, and its purity confirmed by high performance liquid chromatography. The compound was dissolved in DMSO (CryoSure–DMSO; Wak Chemie Medical GmbH, Steinbach, Germany) to the final concentration of 20mM and stored frozen at -80°C. HU was obtained from Sigma-Aldrich (St Louis, MO, USA).

Cells and cell culture

The JAK2^{V617F} erythroleukemia cell lines HEL (DSMZ, Germany) and UKE1 (a kind gift of Dr. W. Friedler, Eppendorf Hospital, Hamburg, Germany) were grown in RPMI1640 medium (Cambrex Bio Science, Verviers, Belgium), supplemented with 10% fetal bovine serum (Euroclone, Wetherby, West Yorkshire,UK), 2mM glutamine (Euroclone) and 110 uM gentamycin (PHT Pharma, Milano, Italy), and in the case of UKE1 with 10% horse serum (GIBCO Invitrogen Corp., Paisley, United Kingdom), 10 μM hydrocortisone and 25 mM HEPES buffer solution, both obtained from Sigma-Aldrich (St Louis, MO).

Peripheral blood was collected in EDTA tubes from MPN patients bearing the JAK2^{V617F} mutation after informed consent, as approved by the Internal Ethical Committee. The mononuclear cells were separated by standard Ficoll-Hypaque gradient centrifugation.

Colony forming assays

Colony forming assays were performed by plating $3x10^5$ mononuclear cells in warm methylcellulose medium (HSC-CFU complete medium including recombinant human erythropoietin, Miltenyi Biotech, Bergisch, Gladbach, Germany) in the presence of increasing concentrations of GVS and HU. Cells were incubated for 10-14 days at 37°C and 5% CO_2, and colonies were counted using an inverted microscope (Axiovert 40 CFL, Zeiss Goettingen, Germany).

Alamar blue cytotoxicity assays

Cytotoxicity assays were performed using the alamar blue vital dye essentially as described [12]. Briefly, cell lines were plated at 10^4 cells/well in the absence or presence of increasing concentrations GVS and/or HU. In particular, for Chou Talalay analyses of synergism, cells were treated with six doses of each drug, i.e. GVS 20-120 nM and HU 200-1200 μM at a constant ratio of 1:10000 for HEL and GVS 13.3-116 nM and HU 20-175 μM at a constant ratio of 1:1500 for UKE1. After 48 hours of culture, 1/10 volume alamar blue solution (Biosource International Inc., Camarillo, CA) was added, and after further 8 hours incubation, the plates were read in a fluorimeter (Tecan Austria GmbH, Salzburg, Austria) with excitation at 535 nm and emission at 590 nm. The combination index (CI) for each drug combination was calculated by median dose effect analyses [19] using the CI equation within the commercially available software (Calcusyn; Biosoft, Cambidge, UK). CI values of less than 1.0 represent synergism of the 2 drugs in combination, CI of 1 indicates an additive effect and CI >1 antagonism.

Apoptosis measurement

Cells were plated at $2x10^5$/ml and treated with GVS and HU, alone or in combination. After 24 hours treatment, cells were stained with 7-Aminoactinomycin D (7AAD, BD Biosciences) and Syto-16 (Invitrogen srl, San Giuliano Milanese, Italy), according to manufacturer's instruction, and the percentage of apoptotic and necrotic cells was determined by standard flow cytometry on a FACscan Instrument (BD Biosciences). Syto16+ 7AAD- cells represent live cells, syto16- 7AAD- apoptotic cells and syto16- 7AAD+ cells necrotic cells [20].

Cell cycle fraction analysis

For cell cycle analysis, washed cells were incubated with 5 μg/mL propidium iodide (PI, Sigma-Aldrich, Milan, Italy) dissolved in PBS with 0.2 mg/mL RNase A and 0.15% NP40 (Sigma-Aldrich) incubated overnight at 4°C. Analysis was performed on a FACSscan Instrument using the ModFit LT software (Verity Software House, Inc., Topsham, ME, USA).

For combined analysis of DNA content and bromodeoxyuridine (BrdU) incorporation, cells were first incubated for 60 min with 20 μM BrdU (Sigma-Aldrich, Milan, Italy) at 37°C, harvested and fixed overnight with 70% ethanol at 4°C. After washing, the cells were treated with 2N HCl containing 0.5 % Triton-X (Sigma-Aldrich, Milan, Italy) for 30 min at room temperature (RT). The cells were washed with PBS containing 0.2% Tween-20 and 0.5% BSA, and incubated with FITC anti-BrdU antibodies (BD Biosciences, Faranklin Lakes, NJ) followed by FITC-conjugated rabbit-mouse (DAKO Italia, Milan, Italy) for 20 minutes at room temperature and

then 5 μg/ml PI and RNase (10 μg/ml) (Roche) solution overnight at 4°C. The cells were analyzed by flow cytometry (FACScan, BD Biosciences).

Western blot analysis

For protein expression analyses cells were collected, washed in saline and lysed in M-PER extraction reagent (Pierce, Rockford, IL) according to the manufacturer's protocol. Protein concentration was determined by the Bradford assay (Bio-Rad, Hercules, CA, USA). Equal amounts of protein were separated by electrophoresis on a sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane (Pierce, Rockford, IL). Western blot analysis was performed using the following antibodies: JAK2, STAT5, caspase 3 and β-actin from Santa Cruz (Heidelberg, Germany); p21CDKN1A and phosphorylated JAK2 (Tyr1007/1008), STAT5 (Tyr694) from Cell Signalling Technology (Danvers, MA, USA). Detection was performed using horseradish peroxidase-labeled secondary antibodies (Santa Cruz) and Super Signal West Pico Chemiluminescent Substrate (Pierce).

Statistical analysis

The Student's T-test was used to compare experimental groups, with a p value of <0.05 considered statistically significant. The effectiveness of various drug combinations was analyzed by the method of Chou and Talalay [21]. The combination indices were calculated using the Calcusyn Software (Biosoft, Cambridge, UK).

Results

Synergistic induction of cytotoxicity and apoptosis in JAK2^{V617F} cells by Givinostat and Hydroxyurea

In order to assess the effect of combining GVS and HU in MPN, the JAK2^{V617F} HEL cell line was treated for 48 hours with suboptimal doses of either drug individually or in combination, and cytotoxicity was analyzed with the alamar blue vital dye. As shown in Fig.1A, combined treatment of HEL with 25 nM GVS and 1 mM HU yielded growth inhibition more than the sum of either agent alone. Indeed GVS and HU alone induced 6.8% and 42.4% of cytotoxicity respectively, whereas both agents together induced 56.6% cell death, compared to 49.4% for a simple additive effect ($p<0.05$). In order to extend these data to a second cell line, the same experiment was performed with the JAK2^{V617F} UKE1 cell line. Treatment of UKE1 cells with 100 nM GVS and 0.15 mM HU in combination was more cytotoxic (75.3%) than the sum of cytotoxicity of each agent alone (20.8% and 35.7% giving a sum of 56.5%)($p<0.05$) (Fig.1B).

In order to strengthen the validity of these preliminary data, dose response curves for each drug were performed for each cell line individually. Examples of the dose response curves obtained for UKE1 are shown in Supplementary Fig.1. A range of six drug doses were then chosen for each line to perform analyses of synergism according to the method of Chou and Talalay, i.e. doses of drugs that cover both curves and are used at a fixed ratio to each other. The chosen doses were 20-120nM and 13-116 nM GVS, and 200-1200 μM

and 20-175 μM HU, for HEL and UKE1, respectively, with fixed ratios of GVS:HU of 1:10000 and 1:1500 for the two cell lines, respectively.

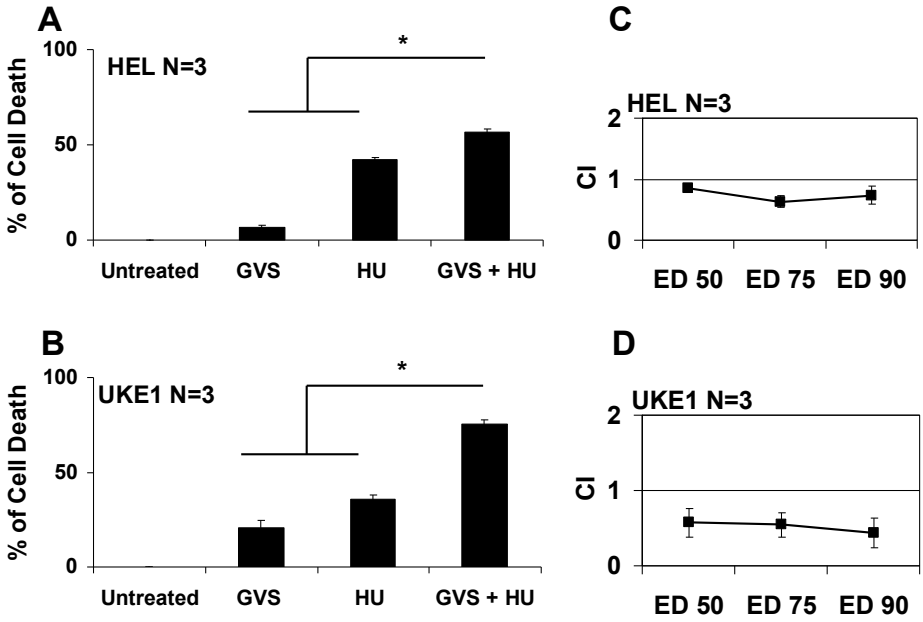


Figure 1. Combined treatment of GVS with HU synergistically induces cell death in HEL and UKE1.

Panels A and B: HEL (A) and UKE1 (B) cell lines were exposed to the 25 or 100 nM GVS and 1 or 0.15 mM HU, respectively, alone or in combination and viability was determined with the alamar blue dye. The data are the means and standard deviations of three experiments. *: $p < 0.05$ by Student's T test for the comparison between the sum of the effect of each drug alone compared to the effect of the drugs combined.

Panels C and D: The dose response curves were performed according to the Chou Talalay method with fixed molar ratios of GVS to HU of 1:10000 and 1:1500 for HEL (C) and UKE1 (D), respectively. Combination index values (CI) were calculated with the Calcusyn software. The data shown are the mean CI values and standard deviations for the ED_{50}, ED_{75} and ED_{90} effective

doses, obtained from three separate experiments for each cell line. CI<1.0 indicates synergism CI=1.0 indicates an additive effect and CI >1.0 antagonism.

The cells were tested with these six doses of drugs alone or together and combination indices (CI) at different effect levels were calculated with the Calcusyn software. The results of three independent experiments demonstrate a combination index (CI) of <1 for both cell lines (range 0.63-0.84 for HEL and 0.43-0.57 for UKE1), at effective doses (ED) ranging from ED50 to ED90 (Fig. 1C and D and Supplementary Fig.1). These data thus confirm the synergistic effect of GVS and HU in these two cell lines.

We also evaluated whether synergism was measurable in terms of apoptosis induction, measured at 24 hours. Both the HEL and UKE1 cell lines were treated with suboptimal concentrations of either drug alone or in combination and cell death was measured by 7AAD and syto16 staining and flow cytometry. Syto16-7AAD$^-$ are apoptotic cells whereas syto16-7AAD$^+$ cells are necrotic [20] and total cell death was considered for the analysis of both cells lines, since necrosis was more rapid in UKE1 than HEL cells (see also Supplementary Fig.2 and 3). As shown in Fig.2A and B and in the representative experiment shown in Supplementary Fig. 2 and 3, the combined treatment of either cell line with GVS and HU resulted in a significantly greater cell death induction than the sum of cell death induced by either compound alone. Indeed in HEL cells single agents induced 20.7% and 26.7% cell death compared to 64% cell death in combination, which is more than the 47.4% for an additive effect ($p<0.01$)(Fig.2A). For UKE1, single drugs induced 7.8% and 20.4% cell death and 35.8% in combination which is more than the expected

28.2% for an additive effect ($p<0.05$), confirming the alamar blue results (Fig. 2B).

We conclude that GVS and HU are synergistic at apoptosis induction.

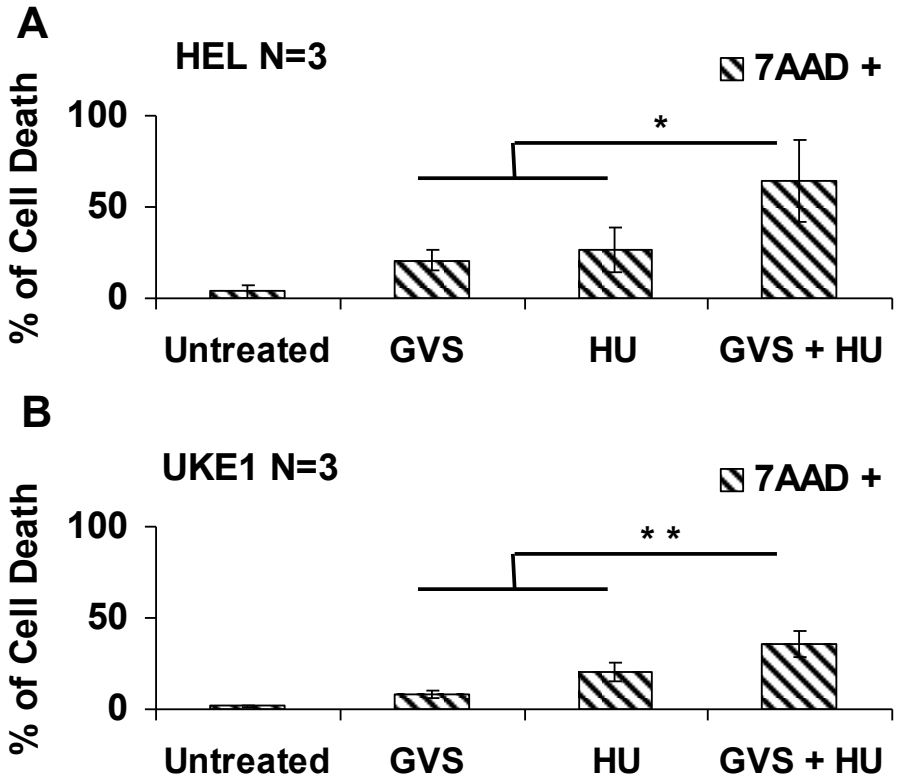


Figure 2. GVS and HU are synergistic for apoptosis induction.

The HEL (A) and UKE1 (B) cell lines were cultured for 24 hours in the presence of 250 or 100 nM GVS and 1.5 or 0.5 mM HU, respectively, either individually or in combination. Apoptosis was determined by 7AAD/Syto-16 staining and flow cytometry. The results are the mean total cell death (apoptosis and necrosis after substraction of background) and standard deviations from three separate experiments. Background in absence of drug treatment was a mean of 3% and 4% for HEL and UKE1, respectively.

Original dot plots of one representative experiment are shown in Supplementary Fig.1. *: $p < 0.05$ and **.$p<0.01$ by Student's T test for the comparison between the sum of the effect of each drug alone compared to the effect of the drugs combined.

Givinostat induces a block in G1 phase of the cell cycle in presence or absence of HU

HDAC inhibitors have been shown to generally induce a block in G1 whereas HU arrests cells in S phase. We therefore analyzed in more detail the effect on the cell cycle of these two agents alone or in combination. As shown in Fig.3 for HEL, GVS alone induced an increase in G1 (from 51 to 63%) and a reduction in S phase cells (from 21 to 15%), as detected by BrdU and propidium iodide staining of DNA. In contrast HU treatment induced a marked increased in S phase (from 21 to 64%). The combination of both drugs blocked live cells in G0/G1 which reached 72% and also induced apoptosis (Fig.3 and Fig.2, respectively). These data suggest that the block in G1 induced by GVS overrides that induced in S phase by HU, and results in increased apoptosis. Similar data were obtained for UKE1 as shown in the representative experiment of Supplementary Fig.4.

We conclude that GVS overrides the S phase block induced by HU and thus increases apoptosis of HU treated cells.

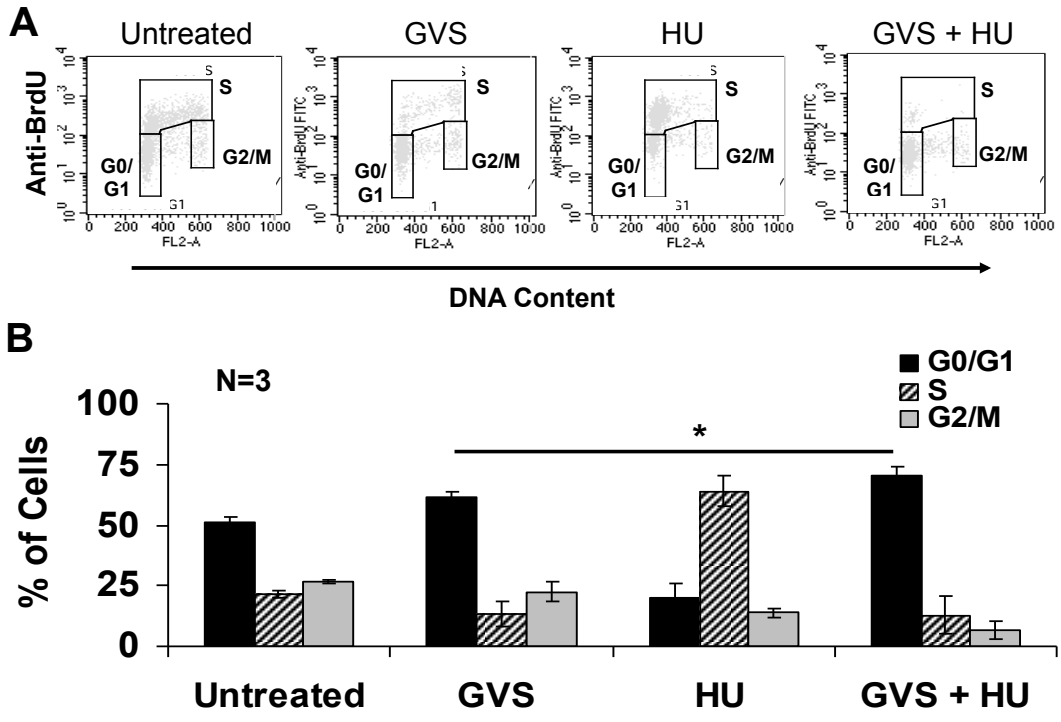


Figure 3. Cell cycle analysis of GVS and HU treated cells

HEL cells were treated for 24-36 hours with the 250 nM GVS and 1 mM HU, alone or in combination, and stained with BrdU and 7AAD followed by flow cytometry (panel A). Cells were gated on live singlet events before analysis to evidence live cells. Panel A present the histograms and dot plots of one representative out of three experiments. Panel B reports the mean percentages of cells in the different phases and standard deviations of three experiments.

Lack of p21CDKN1A induction by Givinostat in presence of HU

In order to investigate the possible mechanism of action of the synergistic effect of GVS and HU in JAK2^{V617F} cells, we investigated known protein modulations induced by these drugs. GVS alone is

known to inhibit JAK2 and STAT5 phosphorylation [12]. This was confirmed here by Western blot analysis (Fig.4A and B). HU also reduced these phosphorylated proteins and the combined use of both drugs was most effective (Fig.4A and B). Both drugs together had also some effect on total STAT5 protein. Active caspase 3 was induced by either drug alone in HEL, although this was not detectable in UKE1, but the combined presence of both compounds induced even the strongest caspase 3 activation in both cell lines, that paralleled the decreased pro-caspase 3 (Fig.4A and B). Interestingly, the cyclin dependent kinase inhibitor p21CDKN1A, an inhibitor of caspase 3, was induced by GVS alone, but not HU. Furthermore the addition of HU in presence of GVS abolished the p21CDKN1A induction normally observed in presence of HDAC inhibitor.

We conclude that the combination of GVS and HU leads to a strong inhibition of the JAK2-STAT5 pathway and high caspase 3 activation in absence of p21CDKN1A induction.

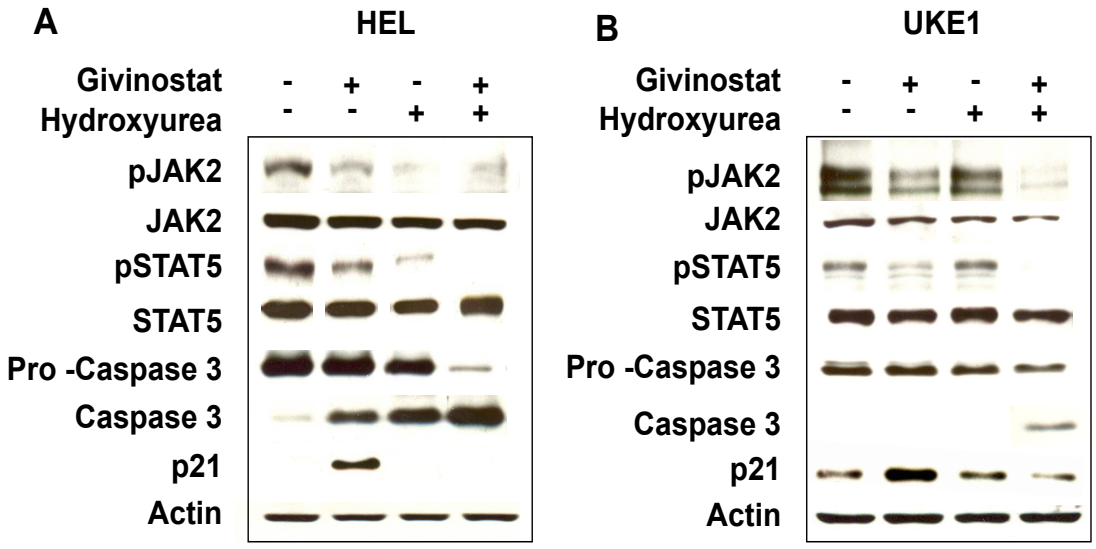


Figure 4. Pattern of protein expression modulated by GVS and HU.
HEL (panel A) and UKE1 cells (panel B) were treated for 24 hours with 250 nM or 100 nM for GVS and 1.5 mM or 0.5 mM for HU, respectively, and cell lysates were prepared and analyzed by Western blotting using the indicated antibodies. The results shown are representative of at least three separate experiments.

Givinostat and HU are at least additive in colony assays using MPN patients cells

Having established a synergistic activity of GVS and HU in two cell lines, we investigated the activity of GVS and HU in colony assays using freshly isolated mononuclear cells from $JAK2^{V617F}$ positive patients. In this case low doses of drugs were used since patient cells are highly sensitive to the compounds in colony assays [12]. As shown in Fig.5A, 10 nM GVS induced a 42.6% reduction in colonies, whereas 6 μM HU reduced colony formation by 61.1%. Both drugs together had an approximately additive effect with 99% reduction in colony number. Similarly in further 5 patients samples tested, 10 nM GVS induced a 37% reduction in colonies, whereas 3 μM HU reduced colony formation by 53.1%. Both drugs together had an approximately additive effect with 92% reduction in colony number.

Altogether these data confirm the favourable association of these two drugs in primary MPN cells. We also attempted to measure p21CDKN1A induction in patients purified $CD34^{+}$ cells, but could not detect the protein in any conditions used, probably due to the limited amount of material available.

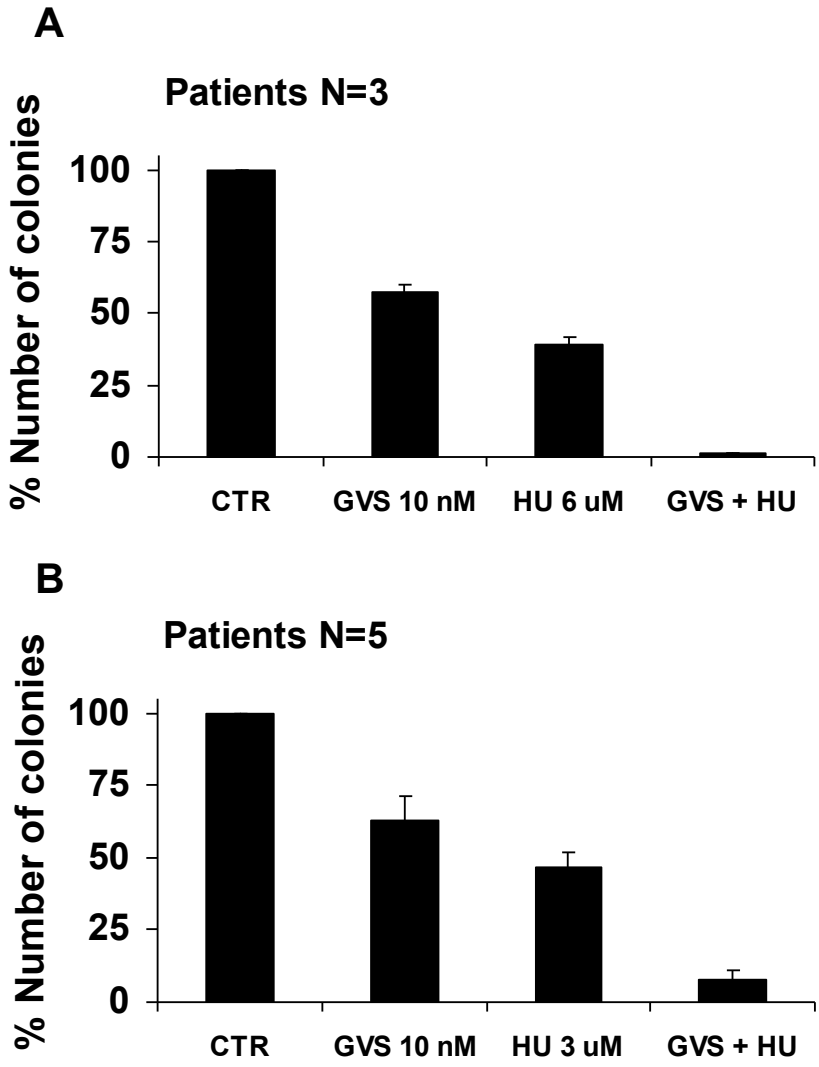


Figure 5. Effect of GVS and HU on colony formation by freshly isolated JAK2^{V617F} patients cells.

Colony-forming assays were performed with peripheral blood mononuclear cells isolated from eight MPN patients bearing JAK2^{V617F}, plated in presence or absence of 10 nM GVS and either 6 μM HU (panel A) or 3 μM HU (panel B), alone or in combination. The data presented are the means and standard deviations of three (A) or five (B) independent experiments using different donors.

Discussion

In this report we demonstrate that GVS and HU have a synergistic effect on JAK2^{V617F} cell lines in terms of cytotoxicity assays and apoptosis induction and investigate possible mechanisms of such synergy.

We show that these two drugs are synergistic in cytotoxicity assays using two different JAK2^{V617F} cell lines, using the Chou Talalay method [22]. Synergy was confirmed at the level of apoptosis induction. More in depth, analysis of cell cycle phases showed that GVS blocked JAK2^{V617F} cells in G1 phase. In contrast HU alone induced S phase arrest, as expected [9]. When both drugs were present a G1 block was still observed, but cells underwent rapid apoptosis. Thus the two drugs sensitized each other to apoptosis induction.

We then analyzed several proteins involved in regulating cell cycle progression and apoptosis in JAK2^{V617F} cells. We show that both GVS and HU inhibited JAK2 and STAT5 phosphorylation with minor effects on total protein levels. Combination of both drugs on these proteins seemed to be at least additive on STAT5 phosphorylation and also reduced total STAT5 protein. Similarly caspase 3 was activated strongly by either drug alone but their combination was synergistic, in support of their effect on apoptosis induction. p21CDKN1A CDK inhibitor was induced by GVS, a phenomenon expected for this class of compounds. Indeed p21CDKN1A induction is almost universally observed in response to different HDAC inhibitors and in a variety of tumour cell lines [14-16]. This takes place at the transcriptional level and is thought to be a direct result of

modification the histone acetylation by GVS [15, 23]. Most interestingly p21CDKN1A induction was not observed in presence of HU, either alone or combined with GVS. Thus the presence of HU blocked p21CDKN1A induction, even though the cells were at least temporarily arrested in G1. This observation suggests that that the lack of p21CDKN1A induction may be at the basis of synergism of the two drugs. Indeed p21CDKN1A is induced by different compounds in a p53-dependent or independent manner. This CDK inhibitor blocks cells in the G1/S boundary, as reviewed in [24]. GVS and other HDAC inhibitors have been shown to block cells in G1, at least in part through p21CDKN1A induction.

Furthermore p21CDKN1A has been observed to protect cells from apoptosis in most cellular contexts investigated, including chronic myeloid leukemia cells and myelomonocytic cells following HDAC inhibitor treatment [25-28], reviewed in [29]. Thus a lack of p21CDKN1A induction in presence of HU may at least in part explain the increased apoptosis observed in the presence of both compounds. The mechanism by which p21CDKN1A inhibits apoptosis is not completely clear and may include binding to and inhibiting pro-caspase 3, as well as the pro-apoptotic kinases ASK1 and JNK [30, 31], the apoptosis inhibitor protein cIAP1 or cytoplasmic CDK2 [29, 30, 32]. Caspase 3 itself may regulate p21CDKN1A by cleaving its N-terminus and this appears to be an important mechanism of the apoptotic response to different agents [33-35]. Thus p21CDKN1A and caspase 3 interact with each other and have opposite functions with regard to apoptosis.

Our observations that GVS and HU combination led of decreased p21CDKN1A induction and increased caspase3 activation

in parallel with increased apoptosis is consistent with the anti-apoptotic activity of p21CDKN1A. A decreased p21CDKN1A induction has similarly been suggested as the basis for synergism of another HDAC inhibitor with HU in melanoma cells [18] and with PI3 kinase inhibitors in myeloid leukemia cells [36]. The suggested mechanism of GVS and HU synergistic activity though JAK2-STAT5 inhibitions and p21CDKN1A/caspase 3 modulation is schematized is Fig.6.

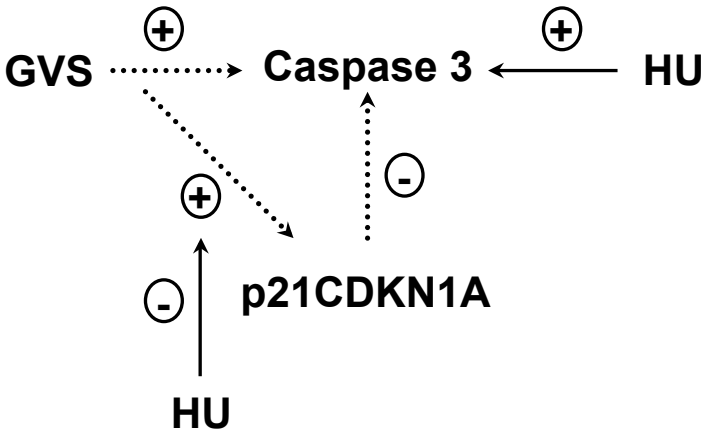
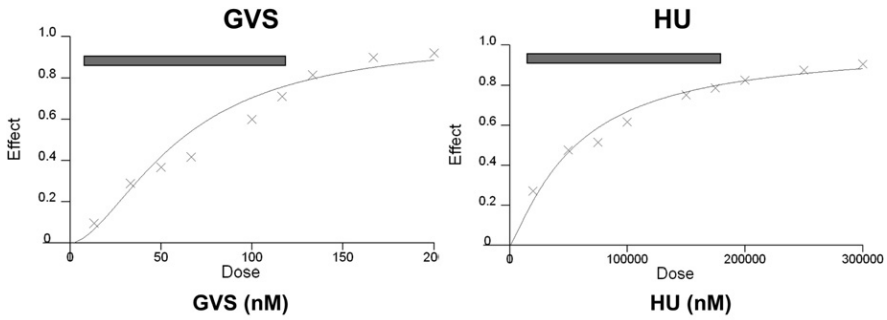


Figure 6. Schematic representation of GVS and HU effects.
GVS inhibits the JAK2-STAT5 pathway and upregulates p21CDKN1A and caspase 3. p21CDKN1 is an inhibitor of caspase 3. HU induces caspase 3 and inhibits p21CDKN1A induction by GVS. These effects may be the molecular basis for GVS and HU synergism.

A promising additive effect of GVS with HU was observed on colony growth of $JAK2^{V617F}$ cells isolated from MPN patients. The fact that synergism was not observed with patients primary cells, in

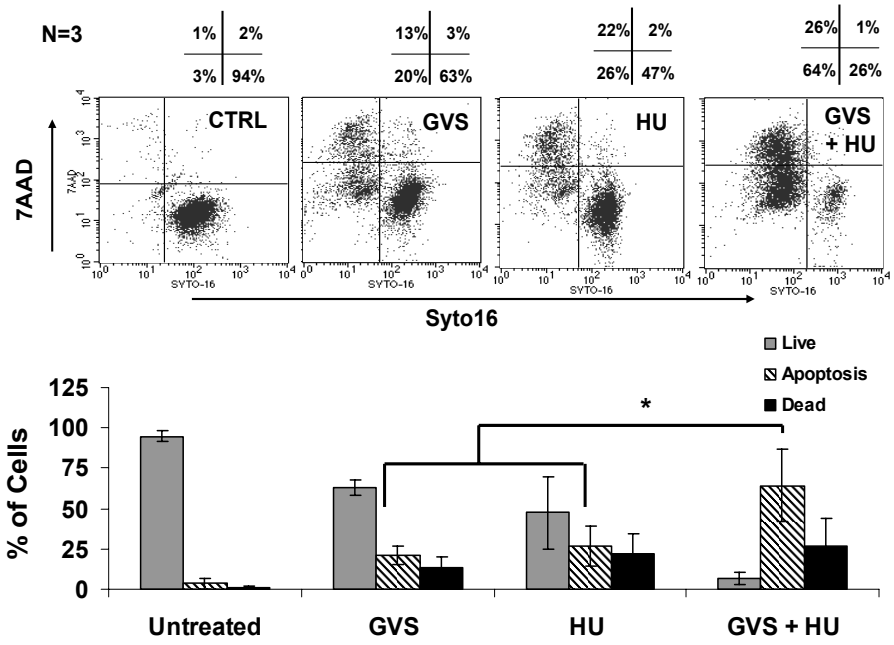
contrast to the cell line data, may have been due to the type of assays that was feasible with patients cells (colony assays), or to the fact that only a proportion of patients $CD34^+$ cells bear the JAK2 mutation. The additive effect does in any case support the combination of these two agents in the clinic. GVS is good candidate drug for MPN patients since this drug has been shown to be particularly active in $JAK2^{V617F}$ cells [12, 19]. The specificity of GVS appears to be due to its ability to inhibit of $JAK2^{V617F}$ and downstream STAT5/STAT3 phosphorylation [12, 19]. More recent studies indicate that GVS acts through modulation of both JAK-STAT dependent and independent genes [13]. On the basis of these in vitro data, the drug has recently been tested in a pilot clinical study for $JAK2^{V617F}$ positive MPN as single treatment [10]. The results on a limited number of patients have shown that the drug is active in this setting, diminishing pruritus significantly in most patients and reducing splenomegaly in 75% of PV/ET patients. Furthermore a trend in the reduction of the $JAK2^{V617F}$ allele burden was observed in these patients, with an overall satisfactory safety profile [10]. HU is a standard drug for PV making it a choice for combined treatment with GVS [1, 37-39]. The concentrations of drugs used here, particularly on primary MPN cells are clinically achievable [40]. A randomized Phase II clinical trial of GVS and HU has recently been completed and has indicated a favourable clinical activity of the combination of these two compounds in the treatment of PV patients [41].

Supplementary Figure E1. Dose response curves of GVS and HU in UKE1 and combination indices (CI) obtained in 3 experiments.

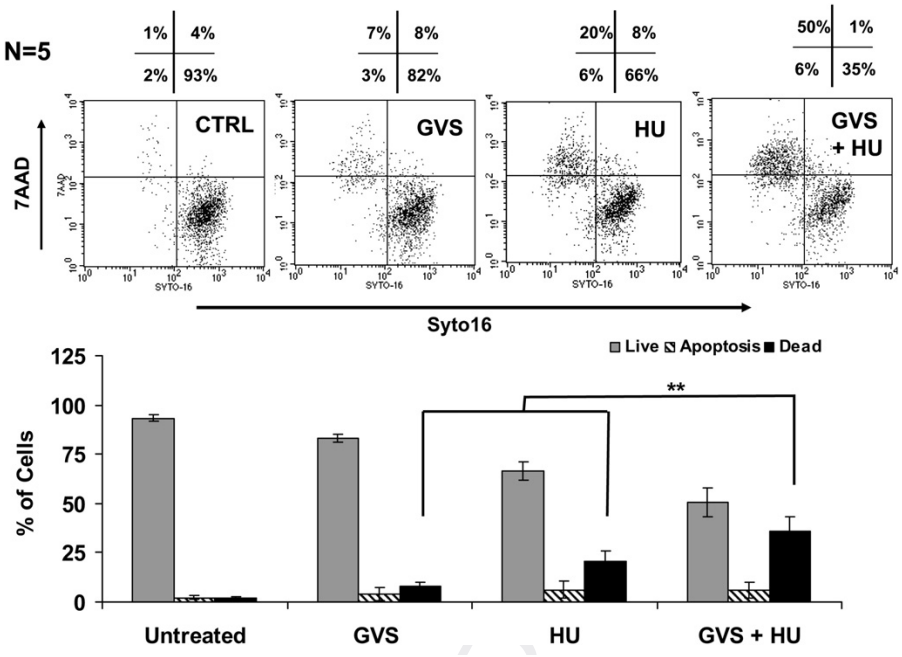


Dose level	Exp. 1 CI	Exp. 2 CI	Exp. 3 CI	Mean CI
ED 50	0,8	0,5	0,4	0,56
ED 75	0,7	0,5	0,4	0,53
ED 90	0,6	0,4	0,3	0,43

Supplementary Figure E2. 250 nM GVS and 1.5 mM HU are synergistic for apoptosis induction of HEL.



Supplementary Figure E3. 100 nM GVS and 0.5 mM HU are synergistic for apoptosis induction of UKE1.



Supplementary Figure E4. Standard cell cycle analysis of UKE1.

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Chapter 4

4.1 Summary

Histone deacetylation inhibitor (HDACi) have recently been proposed as new anti-cancer agents in a variety of conditions. Indeed, due to their ability to selectively induce cell cycle arrest, differentiation and/or induce tumor cell death in vitro and in vivo, while showing little toxicity to normal cells, these compounds are attractive chemo-therapeutic agents[1-4]. Furthermore the pleiotropic cellular effects of HDACi can act cooperatively to mediate potent antitumor activities; however, the molecular processes underlying the effects of HDACi remain to be fully elucidated. HDACi have been proposed to control gene expression primarily by inhibiting the activity of HDACs and thereby shifting the overall balance between histone acetyltransferases (HATs) and histone deacetylases (HDACs) in the cell to promote histone hyperacetylation[5,6;7]. Enhanced histone acetylation of lysine residues negates the positive charge of the amino terminal histone tails, reducing the electrostatic interaction between these histone tails and the negatively charged DNA backbone. Accordingly, this hyperacetylated histone state presents an open and accessible DNA backbone for the transcriptional machinery to interact with, and thus encourages the activation of gene transcription[8]. This is one proposed mechanism of action of HDAC inhibitor (HDACi). It is important to note however that HDACi have been reported to alter the expression levels of only a small percentage of genes and are able to both activate and repress genes. How this is performed is therefore not completely understood. One possible

additional fact is that the activity of HDACs is not exclusive to the transcription machinery; they also regulate non-histone proteins[9,10], giving a new perspective on the mechanistic action of these enzymes, indicating that HDACi could have a much broader effect on cellular physiology than originally understood [11,12].

Our project inserts itself in this context, but is centered on hematological malignancies. In fact, previous data from our laboratory had suggested that JAK2^{V617F} positive myeloproliferative neoplasm (MPN) cells are particularly responsive to this class of drugs, and more specifically to Givinostat (GVS), a pan-HDACi[13-16]. Here, we have therefore explored the activities and mechanism of action of GVS in the context of this disease subtype, alone or in combination with a standard drug for this disease, hydroxyurea (HU).

In the first part of this work we have investigated the biological effect and mechanism of action of the HDACi GVS in JAK2^{V617F} cells. The JAK2^{V617F} cell lines HEL, UKE1 and SET2 showed, in cytotoxicity, proliferation and colony assays, an IC_{50} 2-3 fold lower than that generally observed for other leukemic cell lines bearing JAK2 wild type (wt). Furthermore our data determined that the increased sensitivity of the JAK2^{V617F} cells was due to both a block of proliferation in G1 phase and induction of apoptosis at lower drug doses in JAK2^{V617F} cells with respect to JAK2wt cells.

In order to analyze the mechanism of action of GVS in JAK2^{V617F} cells at a molecular level, we initially performed Western blot analyses of the JAK2-STAT5-ERK1/2 pathway, known to be hyperactivated by the JAK2 mutation in these cells and probably at the basis of the disease. Protein phosphorylation assays of the JAK-

STAT pathway in JAK2^{V617F} cell lines showed that GVS in all cases inhibited JAK2 and downstream STAT5 and ERK1/2 phosphorylation. In contrast, GVS did not fully inhibit the JAK2-STAT5-ERK1/2 pathway in JAK2wt cells. The confirmed lack of phospho-STAT5/ERK1/2 inhibition in JAK2wt leukemic cells is probably due to JAK2- independent mechanisms of STAT5/ERK1/2 activation present in these cells. We propose therefore that inhibition of the JAK2-STAT5-ERK1/2 pathway by GVS in JAK2^{V617F} cells is in part responsible for growth inhibition and apoptosis induction induced by GVS in these cells (Figure 15).

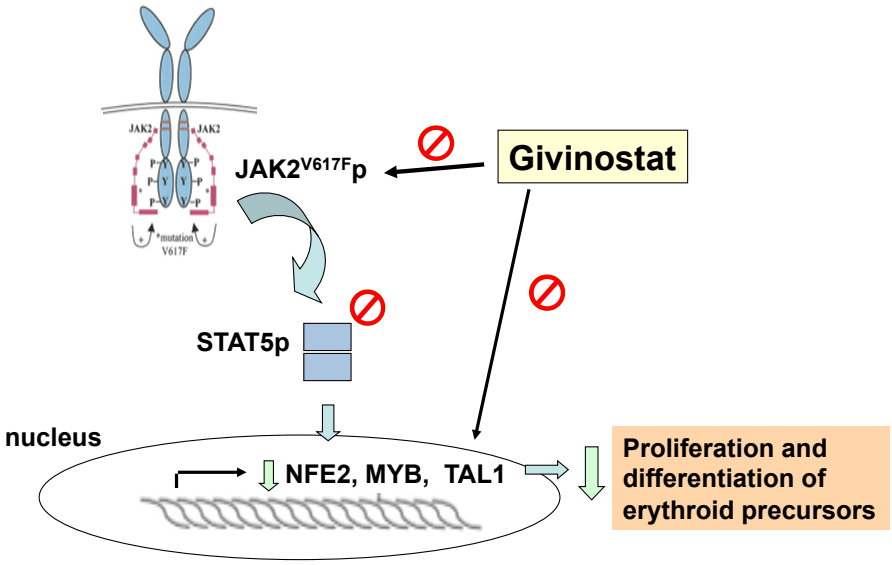

Figure 15. MPN cells are highly dependent upon JAK2-STAT5 signalling pathway

In order to understand in more details the molecular consequences of GVS treatment in MPN cells, we also performed gene expression profiling by whole genome microarray analysis. This

study revealed 293 genes commonly modulated by GVS in JAK2^{V617F} cells. Functional analysis of the modulated genes showed that 19 genes were related to cell cycle control. In addition, we found that 33 GVS - modulated genes were associated with hematopoiesis. Of most interest were NF-E2 (Nuclear Factor-Erythroid-derived 2), C-MYB, and TAL1 genes, all of which encode transcription factors known to control differentiation along the erythro-megakaryocytic-myeloid lineages, the lineage specifically deregulated in MPN

We could confirm by RTQ-PCR that these three genes were modulated at the RNA level by GVS. At the protein level, we could demonstrate significant downmodulation of C-MYB and NF-E2 in all three JAK2-mutated cell lines, confirming the messenger RNA data. In contrast, we observed only a marginal downmodulation of TAL1 protein. This suggests that the TAL1 messenger RNA decrease after GVS treatment might not be sufficient or protein stability too high to lead to significantly decreased TAL1 protein expression.

One of the most interesting identified target of GVS in the context of MPN was NF-E2. Indeed this transcription factor had been shown previously by other groups to be overexpressed in the vast majority of patients with polycythemia vera (PV) (over 90%)[17,18]. In PV bone marrow, NF-E2 is overexpressed in megakaryocytes, erythroid and granulocytic precursors[19]. Many other observations reveal that NF-E2 is a promising candidate for the molecular etiology of PV. For example the laboratory of Dr. Pahl has previously reported that NF-E2 overexpression delays erythroid maturation and retains erythroid cells in the proliferative stage of development for an extended time[20,21]. Thus NF-E2 expression in PV may be at least one of the elements at the basis of the phenotype of the disease[22,23]. It was

also reported that the degree of NF-E2 overexpression correlated with the JAK2 genotype and the percentage of JAK2^{V617F} mutant allele expressed. However, whether NF-E2 overexpression is mediated by the JAK2^{V617F} mutation, or whether it is the result of independent alterations is currently being investigated, but several lines of evidence suggest that NF-E2 overexpression may be independent of the presence of the JAK2^{V617F} mutation. Indeed in our study, the specific JAK2 inhibitor TG101209 did not inhibit NF-E2 protein expression to a significant levels at active doses, suggesting that in MPN cells, NF-E2 expression is mostly independent from JAK2 activity.

The C-MYB transcription factor modulated by GVS is another important target molecule in the context of MPN[24]. Indeed C-MYB is required at multiple stages of hematopoiesis, including long term self-renewal of hematopoietic stem cells, fetal erythropoiesis and commitment towards the erythroid at the expense of the monocyte and megakaryocytic lineages. Furthermore C-MYB functional mutants show a myeloproliferative-like disease[25,26]. We showed that GVS modulated expression of C-MYB, like that of NF-E2, independently from the JAK2-STAT5 pathway. Modulation of the NF-E2 and C-MYB proteins by the HDACi, therefore, presumably takes place through modification of histones associated with these gene promoters. Indeed analysis of acetylated histone H3 by ChIP assay of the NF-E2 promoter showed that H3K9ac on both the proximal and distal promoters of NF-E2 was highly enriched in GVS-treated cells compared with controls. In contrast, H3K14ac was decreased by HDACi treatment, although not in a statistically significant manner. The effects were specific and not observed in a

control gene AGTR1, the expression of which was not modulated by GVS in GEP analysis.

These data demonstrate that changes in acetylation state of specific lysine residues of histone H3 take place on the promoters of NF-E2 in response to GVS in parallel with a decreased in its transcription, suggesting a direct effect of GVS on the promoter of this gene, leading to altered expression.

Finally we showed that GVS downmodulated the NF-E2 and C-MYB transcription factors also in JAK2^{V617F} MPN freshly isolated patients samples. The drug inhibited proliferation and differentiation of MPN patients cells toward the erythroid lineage in vitro. Modulation of NF-E2 may contribute to these effects, although other genes, such as C-MYB or others, can also contribute.

We concluded from the first part of our study that downmodulation of NF-E2 and C-MYB may be an important mechanism of action of GVS in MPN, in addition to its inhibition of JAK2 phosphorylation.

The second part of my project was to investigate the possible synergism or antagonism between GVS and other drugs of clinical use for treatment of myeloproliferative neoplasm, in particular Hydroxyurea (HU). HU is an inhibitor of ribonucleotide reductase, holding cells in S phase, induces apoptosis and sensitizes tumours to chemotherapeutics[27]. It is a treatment of choice in MPN[28,29].

Our study showed in 2 different JAK2V^{617F} cell lines (HEL and UKE1) that GVS and HU at low doses potentiated the pro-apoptotic effects of each other, measured both using the vital dye alamar blue as well a standard apoptosis dies measured by flow cytometry. The cytotoxic effect of combining GVS and HU was analyzed for both cell

lines using the rigorous median effect method of Chou and Talalay, obtaining a combination index below 1, which demonstrates a synergistic effect. Also experiments of cell cycle with Bromodeoxyuridine (BrdU) and Propidium Iodide (PI), was realized to study the mechanism of action of each drugs, and their interaction. These experiments showed that HU blocks cells in S phase, GVS in G1, both in presence and absence of HU.

These cell cycle data were put in context with observations at the molecular level. Indeed it is well established that treatment with HDACi induces expression of the cyclin dependent kinase inhibitor (CDKI) p21 in a p53 independent manner, causing G1 arrest, and activates the intrinsic apoptotic pathway. This pathway was therefore analyzed in our cellular context using GCS and/or HU. HU counteracted the induction of p21 by GVS and potentiated caspase 3 activation. Treatment with the two drugs also has an additive effect on the disappearance of total and phosphorylated $JAK2^{V617F}$ in these cells. The modulation of p21 and consequent reduced arrest in G1 may thus explain at least in part the increased apoptosis observed in presence of both compounds, since this CDKI can protect cells from various pro-apoptotic stimuli, in particular by inhibiting caspase 3 (Figure 17).

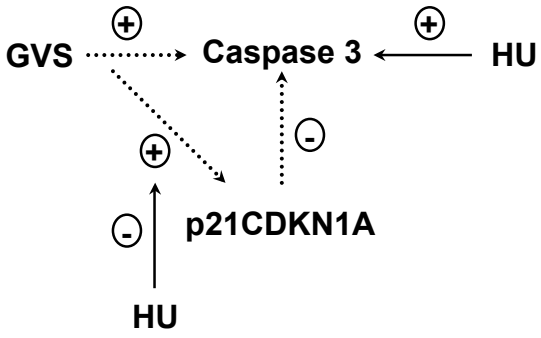

Figure 17. Schematic representation of GVS and HU effects

Finally we could show at least an additive effect of GVS and HU in colony assays using JAK2^{V617F} cells freshly isolated from MPN patients. The additive rather than synergistic effect in this case could be due to the test employed or use of primary cells or other unclear reason. An additive effect is nonetheless a positive result in the context of combined therapeutic strategies.

To conclude, in the second part of the work, we have shown that GVS and HU act in a synergistic manner in inducing apoptosis of JAK2^{V617F} MPN cells at clinically achievable concentrations. These results suggest that the combined application of HDACi and HU is a potential strategy for the treatment of JAK2^{V617F} MPN.

4.2 Conclusions and future perspectives

We have shown in our study that the HDACi GVS is a potent cytotoxic agent against $JAK2^{V617F}$ cells, with some specificity on these cells compared to JAK2 wt leukemia cells. We have shown that the mechanism of GVS in this context includes on the one hand the inhibition of JAK2-STAT5-ERK1/2 phosphorylation in these cells and on the other the downmodulation of the transcription factors NF-E2 and C-MYB in a JAK2 independent manner. Future work along this line of research will be to investigate whether such effects occur also in vivo in MPN patients treated with GVS and whether the proteins identified (JAK2/STAT5 phosphorylation or NF-E2/C-MYB expression) may be used as biomarkers for GVS response in patients.

Another line of research would include a better understanding of the functional consequences of NF-E2/C-MYB downmodulation in MPN cells in vitro and in vivo in murine models, for example using stable or inducible antisense constructs against these specific genes.

Finally this PhD thesis suggests new therapeutic applications of GVS and HU in combination, for the treatment of myeloproliferative neoplasms. This has a direct potential clinical interest, since GVS alone may not be sufficient to obtain an optimal response in PV, ET and MF. It seems more likely that the optimal use of GVS relies upon its combination with other molecules, such as HU, which may synergistically interact and potentiate the pro-apoptotic effects of each other towards cells bearing the $JAK2^{V617F}$ mutation, allowing lower doses of each compounds to be used. Indeed a clinical trial testing this combination has already been implemented[30]. Other strategies to consider in future work may be the combination of GVS

with either JAK2 inhibitors or IFN-alpha, 2 drugs in current clinical trials for MPN and which show promising activities.

In conclusion our study has opended novel therapeutic and research perspectives in the field of MPN

4.3 References

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1- Ariel Amaru Calzada, MD, Katia Todoerti, BSc, Luca Donadoni, BSc, Anna Pellicioli, BSc, Giacomo Tuana BSc, Raffaella Gatta, PhD, Antonino Neri, MD, Guido Finazzi, MD, Roberto Mantovani, PhD, Alessandro Rambaldi, MD, Martino Introna, MD, Luigia Lombardi, PhD, Josée Golay, PhD, *on behalf of the AGIMM Investigators* (2012) The HDAC inhibitor Givinostat modulates the hematopoietic transcription factors NF-E2 and C-MYB in JAK2(V617F) myeloproliferative neoplasm cells. ***Experimental Hematology***, 40(8):634-45.

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