Establishment and application of a highly sensitive coupled luminescent method (CLM) to study natural killer cell cytolytic activity

Dissertation

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Dedicated to my family

TABLE OF CONTENT

1.0 Introduction

Natural killer (NK) cells are white blood lymphocytes of the innate immune system that have diverse biological functions, including recognition and destruction of certain microbial infections and neoplasms [1]. NK cells comprise ~ 10% of all circulating lymphocytes and are also found in peripheral tissues including the liver, peritoneal cavity and placenta. Resting NK cells circulate in the blood, but, following activation by cytokines, they are capable of extravasation and infiltration into most tissues that contain pathogen-infected or malignant cells [2-5]. NK cells discriminate between normal and abnormal cells (infected or transformed) through engagement and dynamic integration of multiple signaling pathways, which are initiated by germline-encoded receptors [6-8]. Healthy cells are protected from NK cell-mediated lysis by expression of major histocompatibility complex (MHC) class I ligands for NK cell inhibitory receptors [6, 9]. The MHC is a group of highly polymorphic glycoproteins that are expressed by every nucleated cell of vertebrates, and that are encoded by the MHC gene cluster. The human MHC molecules are termed human leucocyte antigen (HLA)-A, B and C molecules. Every NK cell expresses at least one inhibitory receptor that recognizes a self-MHC class I molecule. So, normal cells that express MHC class I molecules are protected from self-NK cells, but transformed or infected cells that have down-regulated MHC class I expression are attacked by NK cells [10].

There are 2 distinct subsets of human NK cells identified mainly by cell surface density of CD56. The majority (approximately 90%) of human NK cells are $CD56^{dim}CD16^{bright}$ and express high levels of FcγRIII (CD16), whereas a minority (approximately 10%) are $CD56^{bright}CD16^{dim/-}$ [11]. Resting $CD56^{dim}$ NK cells are more cytotoxic against NK-sensitive targets than $CD56^{bright}$ NK cells [12]. However, after activation with interleukin (IL)-2 or IL-

12, CD56^{bright} cells exhibit similar or enhanced cytotoxicity against NK targets compared to CD56^{dim} cells [12-14].

1.1 Regulation of NK cell function

The functions of NK cells are regulated by a balance of signals (Fig. 1.1). These are transmitted by inhibitory receptors, which bind MHC class I molecules, and activating receptors, which bind ligands on tumors and virus-infected cells [15]. These receptors are completely encoded in the genome, rather than being generated by somatic recombinations, like T- and B-cell receptors.

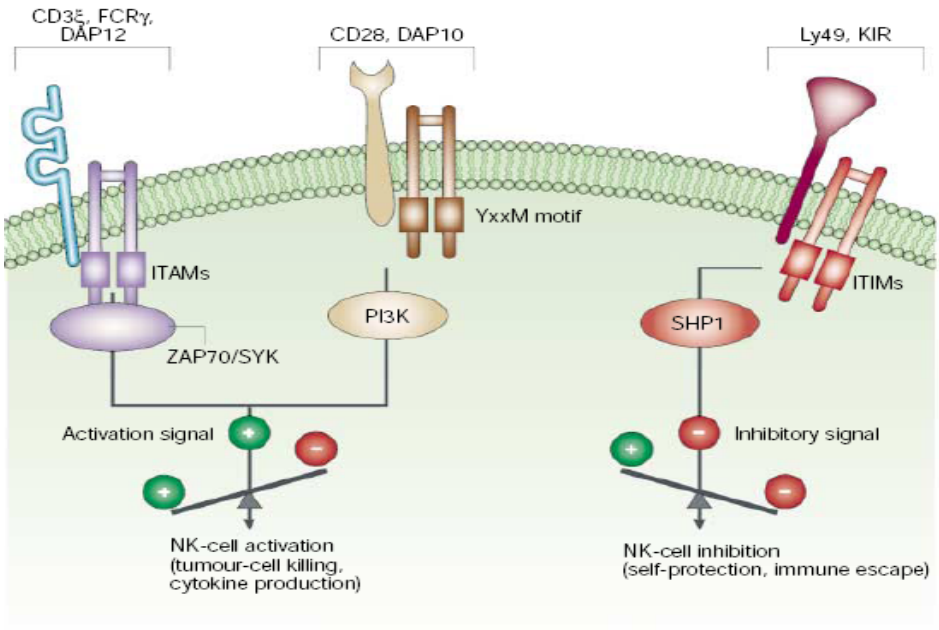


Figure 1.1: Control of NK cell function by the balance of activating/inhibitory signals. NK cell function is regulated by signaling through activation and inhibitory receptors. NK cell activation can occur by means of activating receptors, which signal through ZAP70/SYK when immunoreceptor tyrosine-based activating motif (ITAM)-containing adaptor proteins, such as DAP12, FcRγ and CD3ζ are activated. Alternatively, the CD28 molecule or the lectin-like receptor NKG2D coupled to the adaptor protein DAP10, activates NK cells by associating with phosphatidylinositol-3-kinase (PI3K) through their binding motifs (YxxM motifs). NK cell inhibitory receptors such as Ly49 and KIR contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in

their cytoplasmic domains, which recruit intracellular tyrosine phosphatases such as SHP1 (adapted from reference 10).

The NK cell receptors fall into two main structural classes that each contains activation and inhibitory members. Receptors in the immunoglobulin superfamily include killer inhibitory receptors (KIRs) and leucocyte inhibitory receptors, whereas receptors of the C-type lectin-like family include lymphocyte antigen 49 (LY49), NKG2D and CD94/NKG2 [16-19]. These MHC receptors discriminate among different MHC class I molecules, recognizing specific products that are encoded by individual class I alleles. This is in contrast to the highly conserved NK-cell immunoglobulin (Ig) and lectin-like receptors, which recognize non-classical MHC molecules such as HLA-G and HLA-E. These molecules are considerably less polymorphic, and differ from classical MHC molecules in their patterns of transcription and protein expression, as well as in their immunological functions [20]. An NK cell can express several inhibitory and stimulatory receptors at any one time.

1.2 NK cell inhibitory receptors

Three distinct receptor families that are involved in NK cell recognition of polymorphic MHC class I molecules contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic domains (Table 1.1, Fig. 1.1). Following ligation, these ITIMs become phosphorylated at tyrosine residues, allowing them to recruit the tyrosine phosphatases SHP1, and possibly SHP2. Expression of MHC class I molecules has been shown to protect normal and transformed hematopoietic cells from NK-mediated lysis [21, 22] in several graft versus leukemia (GVL) models. MHC class I expression has also been associated with protection against NK-mediated lysis in patients that have received allogenic bone marrow transplatation [23, 24]. The role of MHC class I molecules in NK cell recognition of solid tumors, by contrast, has been controversial. Disrupting the interaction between human NK cell KIRs and

ligands that are expressed by melanoma cells *in vivo* has been reported to enhance the antitumor responses that are mediated by both innate and adaptive immune effector cells [25]. In the mouse, Ly49C/I inhibitory receptor blockade increased the NK cell-mediated antitumor effects *in vitro* and *in vivo* [26]. Rodent Ly49D [27] also activated the activity of NK cells against hamster CHO tumor cells [28, 29]. Interestingly, MHC class I-specific receptors that inhibit interferon (IFN)-γ production might differ from those that reduce NK cell cytotoxicity [30].

NK cell receptor	**Ligand**
For MHC class I molecules	
Human KIR2DL	HLA-C
Human CD158/KIR3DL	HLA-Bw4, HLA-A
Human CD85i/ILT2	HLA class I
Mouse Ly49	H-2K, H-2D
For non-classical MHC class I molecules	
CD94/NKG2A (mouse: Qa-1b	HLA-E

Table 1.1: NK cell inhibitory receptors (adapted from reference 10)

Human MHC class I molecules are encoded by HLA genes. HLA-E (and its rodent equivalent Qa-1b) binds to signal peptides that are derived from the polymorphic classical MHC class I molecules (such as HLA-A, B and C). This peptide binding requires the activity of the transporter associated with antigen processing (TAP)-1, which stabilizes the HLA-E protein and allows it to be transported to the cell surface. After the HLA-E protein is present on the cell surface, it can interact with CD94/natural killer group 2A (NKG2A) receptors to inhibit

NK cells. Some tumor cells no longer express HLA-A, B and C molecules or TAP, preventing HLA-E from reaching the cell surface. This allows the tumor cell to become vulnerable to NK cell-mediated killing. One *in vitro* study indicated that CD94/NKG2A ligation could inhibit antibody-dependent cellular cytotoxicity (ADCC) that is mediated by NK cells against tumor cell lines [31].

1.3 NK cell activating receptors

NK cells kill certain cancer cell types *in vitro*, even though these cells express significant levels of MHC class I on their surface. Some activating receptors have been implicated in the NK cell recognition of tumors and stressed cells (Table 1.2) [32, 33]. Most of the activating NK cell receptors are transmembrane molecules with short intracellular domains that lack intrinsic signaling activity. They signal by interacting with transmembrane adaptor molecules, such as DAP12, FcRγ and CD3ζ, which contain immunoreceptor tyrosine-based activating motifs (ITAMs) (Fig 1.1).

Receptor	**Species**	**Ligand**	**Signal transduction**
NKG2D	Human Mouse	MIC, ULBP Rae-1, H60	Associated with DAP10 and activates PI3K pathway
NKp30	Human	Unknown	Associated with ITAM-bearing CD3-ζ or FcRγ and activates ZAP70/SYK pathway

NKp46	Human/mouse	Unknown	
NKp44	Human	Unknown	Associated with ITAM-bearing DAP12 and activates ZAP70/SYK pathway
KIR2DS	Human	HLA-C	Same as NKp44
CD94/NKG2C	Human/mouse	HLA-E/Qa-1	Same as NKp44
Ly49D	Mouse	H-2D^{d}	Same as NKp44
Ly49H	Mouse	MCMV m157	Same as NKp44
NKR-P1C (CD161c)	Mouse	Unknown	Same as NKp30
CD16	Human/mouse	IgG	Same as NKp30
Co-stimulatory molecules			
CD28	Human/mouse	CD80, CD86	Activates PI3K pathway
CD27	Human/mouse	CD70	Activates TRAF pathway and ZAP70/SYK pathway
2B4 (CD244)	Human/mouse	CD48	Associated with SAP

Table 1.2: The activation receptors involved in NK cell function. SAP, SLAM-associated protein; SYK, spleen tyrosine kinase; TRAF, tumor-necrosis-factor-receptor-associated factor; ZAP70, zeta-associated phosphoprotein 70 (adapted from reference 10).

1.3.1 Natural cytotoxicity receptors (NCRs).

In vitro NK cell-mediated killing of various cancer cell lines can be blocked by combination treatment with monoclonal antibodies (mAbs) against three receptors that are responsible for the spontaneous cytotoxicity of NK cells. These are NCRs NKp30, NKp44 (only in humans) and NKp46 (in mice and humans) [34-36]. The density of NCRs on the surface of NK cells varies, and there is a direct correlation between NCR expression by human NK cells and their ability to kill tumor cells. NCRs can associate with different signal-transducing adaptor proteins. NK cells from mice with gene disruptions in *DAP12*, *FcRγ* or *CD3ζ* have shown selective deficiencies in their ability to kill certain tumors [37].

1.3.2 Natural Killer Group 2D (NKG2D)

A recently characterized NK cell activating receptor, NKG2D, is a type II disulphide-linked dimer with a lectin-like extracellular domain that is expressed on cytolytic cells of the innate and adaptive immune systems [38]. NKG2D might be expressed as two isoforms (long and short). These forms of NKG2D are associated with the transmembrane adaptor protein DAP10 (NKG2Dlong and NKG2Dshort), which binds to and activates phosphatidylinositol-3-kinase (PI3K) [39], or DAP12 (NKG2Dshort only). Several ligands that bind to mouse and human NKG2D are structurally related to MHC class I molecules, and show diverse expression patterns and modes of induction (Table 1.3) [38, 40-45]. NKG2D recognizes defined antigens that are induced on abnormal cells that are undergoing stress or primary and

secondary tumors [46, 47], and detection of ligands for NKG2D on stressed cells might provide a threshold of activation that allows the killing of abnormal cells.

Ligand	**Expression**	**Induction**	**Structure**
Human			
MICA, MICB, α2	Gut epithelium, epithelial and non-epithelial tumors; tumor cell lines	Heat shock; oxidative stress; tumor transformation; *M. tuberculosis, E. coli* or HCMV infection	Transmembrane protein; MHC class-I-related α1 and domains
UL16 binding protein (ULBP)1,2,3	Tumor cell lines	Tumor transformation	GPI-anchored proteins
Mouse			
Rae-1 family Rae-1α, -1β, -1γ, -1δ	Embryonic tissues; tumor cell lines; not expressed in most normal adult tissues	Retinoic acid; carcinogens; tumor transformation	GP1-anchored proteins; MHC class-I-related α1 and α2 domains
Rae-1ε	Strain-restricted expression		
H60	Strain-restricted expression; activated	Carcinogens; tumor transformation	Transmembrane protein; MHC class-I-related α1 and

	peripheral blood leucocytes and splenocytes		α2 domains

Table 1.3: The ligands for the NKG2D receptor. GPI, gylcosylphosphatidylinositol; HCMV, human cytomegalovirus; MHC, major histocompatibility complex (adapted from reference 10).

Transfection of human MHC class I-bearing targets with MHC class I chain-related molecule A (MICA) rendered these cells susceptible to NK cell-mediated cytotoxicity *in vitro* [38] and expression of H60 or glycosylphosphatidylinositol (GPI)-linked ligands for mouse NKG2D (Rae-1) by MHC class I-expressing tumor cells triggered *in vitro* NK cell and macrophage effector functions [45].

Surveillance that is mediated by NK cell NKG2D might be regulated at the level of ligand availability. As only the long transcript of NKG2D is generally constitutively expressed in naive NK cells and it associates with DAP10, these cells might require secondary signals from other activation receptors, such as NCRs, before they are able to respond to tumors that express NKG2D ligands [48]. A better understanding about the ways in which cells respond to stress (heat shock, viral infection, retinoids, endotoxin and transformation) and signal their stress is required.

1.3.3 NK cell antibody receptors

NK cells also mediate ADCC by expressing a low-affinity Fc receptor for IgG, FcγRIII (CD16). NK cells express different levels of CD16 at various stages of development. The NK cell subset $CD56^{dim}CD16^{bright}$ is responsible for ADCC [49, 50]. When an antibody binds to an antigen, the Fc portion of the antibody is recognized and bound by Fcγ receptors on NK

cells, leading to activation of NK cells and target cell destruction. This ADCC is the dominant component of the activity of antibodies against tumors [10].

1.3.4 NK cell co-stimulatory molecules

The activation of NK cells also results from the action of co-stimulatory molecules. The NK cell activation receptor CD28 interacts with CD80 and CD86 on target cells, which provide co-stimulatory signals for human and rodent NK cells [51, 52]. A proportion of human and most mouse NK cells constitutively express CD27 on their surface [53, 54]. Stimulation of NK cells with CD27 ligand (CD70)-transfected tumor cells could enhance proliferation and IFN-γ production of freshly isolated NK cells in the presence of IL-2. In contrast to signals that are transmitted by CD28 or NKG2D, NK cell cytotoxicity is not induced by CD27 ligation. The NK cell surface molecule 2B4 (CD244) also functions as a co-receptor that is involved in human NK cell activation [55]. 2B4, together with NTBA, are members of the CD2 family. They serve a dual, inhibitory or activating, function depending on the availability of downstream regulating elements in their signaling pathway [56, 57]. Their cytoplasmic portion bind a small cytoplasmic protein termed signaling lymphocyte activation molecule-associated protein (SAP) and delivers triggering signals leading to NK cell activation. The absence of SAP, a molecular defect typical of X-linked lymphoproliferative (XLP) disease, results in binding of SHP-1 phosphatase to 2B4. The consequent SHP-1 activation leads to inhibition of the activation pathways thus blocking NK cell function [58]. 2B4 has been proposed to provide a fail-safe mechanism to prevent killing of normal autologous cells [59]. Interestingly, 2B4 is expressed at the earliert stages of hemopoietic stem cell differentiation, while SAP transcripts are absent [59]. Accordingly 2B4 cross-linking by specific mAbs or by its ligand CD48 inhibits the function of immature NK cells. Remarkably, CD48 is expressed at high densities in bone marrow cells. Thus it is conceivable that 2B4 can provide an

effective fail-safe mechanism to prevent NK cell-mediated damages to bone marrow cells [60]. Other recently identified triggering co-receptors include DNAX accessory molecule (DNAM)-1, NKp80 and CD59. NK cells has also been reported to receive early stimulatory signals directly through binding of the intercellular adhesion molecule (ICAM)-1 to leucocyte function antigen (LFA)-1 expressed on NK cells [61].

1.3.5 Cytokine and chemokine regulation of NK cells

Cytokines are crucial natural adjuvants that are involved in the activation of NK cells against tumor cells. NK cells can be rapidly activated in the periphery by NK cell stimulatory factors, such as IL-12, IFN-α, IFN-β, IL-15 or IL-2. The first indication of their importance came from the observations that virus-induced type I IFNs promoted NK cell-mediated cytotoxicity and proliferation, and promoted innate defence against viral infections [62]. Very little is understood about the IFN and IL-12 signaling pathways in the context of NK cell antitumor activity. IL-2 was the first growth and activation factor that was described for NK cells. The use of this cytokine in the immunotherapy of cancer has met with some success. IL-15 is also a pleiotropic cytokine that is involved in the development and maintenance of NK cells [63, 64]. In NK cells, the IL-15 receptor includes IL-2/IL-15Rβ and –γ c subunits, which are shared with IL-2, and an IL-15-specific receptor subunit, IL-15Rα. While IL-2 is commonly used to activate and expand NK cells *in vitro*, it is conceivable that during the early phases of innate immune responses *in vivo*, IL-15 may exert a predominant role in NK-cell activation and function [65-67].

1.4 Biology of Histone deacetylases (HDACs) and Histone acetyltransferases(HATs)

DNA is packaged into nucleosomes, repeating complexes in chromatin, composed of approximately 146 base pairs of 2 superhelical turns of DNA wrapped around an octomer of

pairs of histones H4, H3, H2a, and H2b. Lysine-rich amino-terminal tails extend from the nucleosomes and are responsible for the conformational changes of the DNA [68-71]. Modification of the histones includes acetylation, methylation, phosphorylation, and/or ubiquitination [70-73]. The “histone code” or “epigenetic code” pertains to the discrete modifications of the histones that regulate chromatin function, including the transcription of genes. The amino acid tails of histones are subject to posttranslational modification by acetylation of lysines, methylation of lysines and arginines, phosphorylation of serines, and ubiquitination of lysines. The most extensively studied posttranslational modifications of histones are the acetylation and deacetylation of the lysine group. The acetylation status of histones and non-histone proteins is determined by HDACs and HATs. HATs transfer acetyl group from acetyl coenzyme A to the N terminal end of lysine tail, neutralizing the charge on the lysine tail and decreasing the attraction between the histones and DNA, thus allowing transcriptional activity [74-77]. HDACs remove the acetyl groups from the lysine residue, restoring the positive charge of the lysine causing condensation of the chromatin, thus leading to repression of gene expression. In humans, 18 HDAC enzymes have been identified and classified, based on homology to yeast HDACs [78-81]. Class I HDACs include HDAC1, -2, -3 and -8, which are related to yeast RPD3 deacetylase and have high homology in their catalytic sites. Recent phylogenetic analyses suggest that this class can be divided into classes Ia (HDAC1 and -2), Ib (HDAC3) and Ic (HDAC8) [82]. Class II HDACs are related to yeast Hda1 and include HDAC4, -5, -6, -7, -9 and -10 [80, 81]. This class is divided into class IIa, consisting of HDAC4, -5, -7 and -9, and class IIb, consisting of HDAC6, and -10, which contain two catalytic sites. All class I and II HDACs are zinc-dependent enzymes. Members of a third class, sirtuins, require nicotinamide adenine dinucleotide (NAD^+) for their enzymatic activity [79]. Among them, SIRT1 is orthologous to yeast silent information regulator 2. Class IV HDAC is represented by HDAC11, which like yeast Hda 1, has conserved residues in the catalytic core region shared by both class I and II enzymes [83].

HDACs are not redundant in function [81, 84, 85]. Class I HDACs are primarily nuclear in localization and ubiquitously expressed, while class II HDACs can be primarily cytoplasmic and/or migrate between the cytoplasm and nucleus and are tissue-restricted in expression.

1.5 HDAC substrates

Recent phylogenetic analyses of bacterial HDACs suggest that all four HDAC classes preceded the evolution of histone proteins [82]. This suggests that the primary activity of HDACs may be directed against non-histone substrates. At least 50 non-histone proteins of known biological function have been identified, which may be acetylated and substrates of HDACs [81, 84-88]. In addition, two recent proteomic studies identified many lysine-acetylated substrates [89, 90]. In view of all these findings, HDACs may be better called '*N*-epsilon-lysine deacetylase'. This designation would also distinguish them from the enzymes that catalyze other types of deacetylation in biological reactions such as acylases that catalyze the deacetylation of a range of N^{α}-acetyl amino acids [91]. Non-histone protein targets of HDACs include transcription regulators, signal transduction mediators, DNA repair enzymes, nuclear import regulators, chaperone proteins, structural proteins, inflammatory mediators, and viral proteins. Acetylation can either increase or decrease the function or stability of the proteins, or protein-protein interaction [86]. These HDACs substrates are directly or indirectly involved in many biological processes, such as gene expression and regulation of pathways of proliferation, differentiation and cell death.

1.6 HDACs and HATs in cancer

Disturbance of the HDAC-HAT dynamic appears to result in the development of cancer. In several hematological malignancies, chromosomal translocations result in the development of fusion proteins, which exert oncogenic effects through interactions with HDACs.

Chromosomal translocations 15;17 and 8;21 produce the fusion proteins PML-RARα and AML-ETO1, respectively. These proteins recruit HDAC-containing transcriptional repressor complexes, resulting in disrupted cell cycle control and the initiation of acute promyelocytic and myeloid leukemias. In the development of diffuse large B-cell lymphoma, BCL6, an oncogenic protein, recruits a HDAC-containing complex to mediate transcriptional repression [92]. Further alterations of the expression and function of HDACs and HATs have been noted in malignancies. Mutations in the HAT p300 and cyclic adenosine monophosphate (cAMP) response element-binding protein are found in patients with Rubinstein-Taybi syndrome, who exhibit developmental delays and increased risk for pediatric malignancies such as neuroblastoma (NB) and retinoblastoma [93]. Overexpression of HDACs has been reported in various solid tumors. Mice lacking the adenomatosis polyposis coli tumor suppressor gene overexpressed HDAC2, which prevented apoptosis in cultured colon cells. Elevated expression of HDAC2 has also been detected at high frequency in human colon carcinomas [94]. Disrupted activities of HDACs and HATs may potentially result in the hypoacetylation of histones in neoplastic cells. Recent evidence suggests that hypoacetylation of lysine 16 of histone H4 may represent a common hallmark or signature of human tumor cells. Analyses of normal tissues, cancer cell lines, and primary tumors indicated that cancer cells predominantly exhibit a loss of acetylated lysine 16 and trimethylated lysine 20 of histone H4. These losses were localized to DNA repetitive sequences that were hypomethylated [95]. These findings implicate the involvement of HDACs in tumorigenesis and emphasize their potential as therapeutic targets.

1.7 Histone deacetylase inhibitors (HDACi)

HDACi are therapeutic agents that inhibit angiogenesis and induce cell cycle arrest, apoptosis and differentiation in cancer cells. They form a new class of antineoplastic agents currently being evaluated in clinical trials for the treatment of various solid or hematologic

malignancies [96-98]. Finnin et al [99] elucidated the crystal structure of the catalytic site of an HDAC homolog and have shown that the direct interaction of HDACi with the active zinc binding site appears to be required to show inhibitory activity (Fig 1.2).

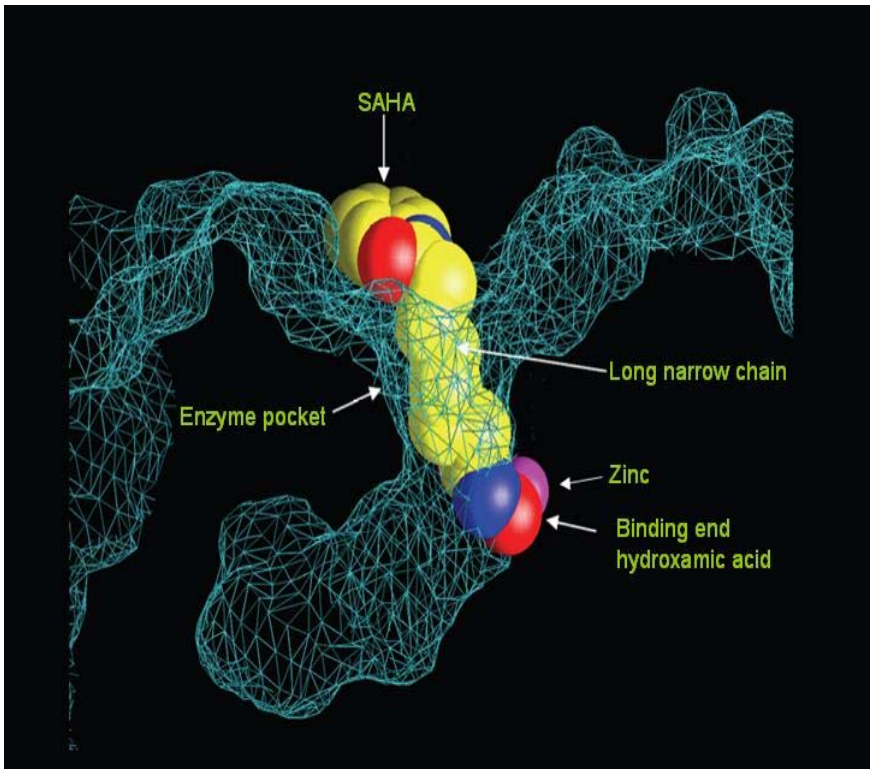


Figure 1.2: Crystal structure showing binding of SAHA on the catalytic site of an HDAC homolog protein. (adapted from reference 99)

Although the HDACi are structurally different, all agents contain a surface recognition site, a metal-binding domain that interacts with the zinc pocket, and a linker domain. Because of the structural differences, the potency and HDAC inhibitory capabilities are variable among classes of HDACi. For example, the enzymatic activity of class III HDACs is not inhibited by compounds such as vorinostat (also called suberoylanilide hydroxamic acid, SAHA) or trichostatin A (TSA) that inhibit class I and class II HDACs [78]. There are 5 classes of HDACi, which include (1) the short-chain fatty acids such as butyrate derivatives and valproic acid (VPA); (2) the hydroxamic acids, which include vorinostat (SAHA), PXD101,

pyroxamide, oxamflatin, cinnamic acid, bishydroxamic acid, LBH589, and scriptaid; (3) cyclic tetrapeptides containing a 2-amino-8-oxo-9, 10-epoxy moiety such as trapoxin A; (4) cyclic peptides that do not have the amino moiety but that include depsipeptide (FK228); and (5) the benzamides such as CI994 and MS 275. Many of these HDACi are in clinical trials both as mono-therapy and in combination with cytotoxic and biologic agents.

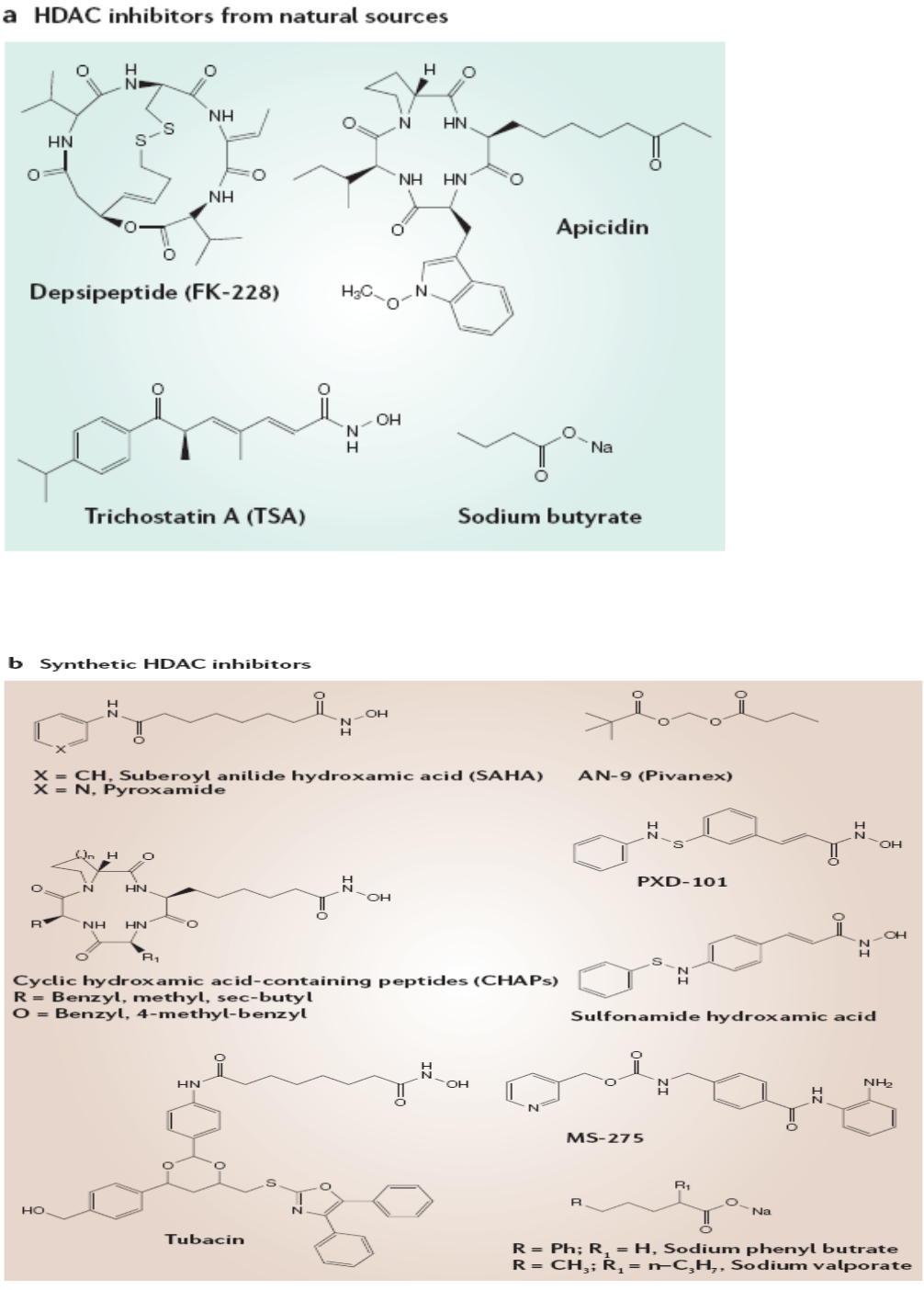


Figure 1.3: Representative structures of the main classes of HDACi either from [a] natural sources or [b] synthetic (adapted from reference 87)

1.8 Biologic activities of HDACi

HDACi have been shown to block the proliferation and the induction of differentiation or apoptosis in a wide variety of transformed cells in culture, including cell lines derived from both hematologic and epithelial derived malignancies. After inhibition of HDAC activity, accumulation of acetylated histones and non-histone proteins is a common upstream event in transformed cells; however, the critical downstream events underpinning the antitumor effects are poorly understood and will depend in part on the HDAC inhibitory spectrum of the specific HDAC inhibitor and resulting substrates of the HDACs affected as well as the cellular context of the tumor. The downstream effects can roughly be divided into 2 broad categories: transcriptional and non-transcriptional effects. Transcriptional and non-transcriptional events can result from the HDACi-mediated accumulation of acetylated histones or non-histone proteins. Recently, evidence has also indicated that HDACi may function to inhibit factors in the angiogenesis pathway [100, 101].

1.8.1 HDACi and cell cycle arrest

HDACi induce an open chromatin conformation through the accumulation of acetylated histones, facilitating the transcription of numerous regulatory genes. Treatment with HDACi results in an increase in expression of cyclin-dependent kinase inhibitor $p21^{waf1}$ and transcriptional repression of cyclin A and D genes, as well as the inhibition of thymidylate synthetase, which is involved in DNA synthesis. These events probably contribute to the cell cycle arrest at G1/S [102-104]. In normal cells, a G2-phase cell cycle checkpoint is activated by treatment with HDACi; however, this checkpoint was found to be defective in several tumor cell lines. Loss of this G2 checkpoint caused the tumor cells to undergo an aberrant mitosis, resulting in fractured multinuclei and micronuclei, and eventually cell death [105]. It is likely that the loss of this G2 checkpoint is somewhat responsible for the selective effects of HDACi treatment in tumor cells [98].

1.8.2 Apoptotic effects of HDACi

HDACi probably induce apoptosis in tumor cells through the activation of numerous mechanisms. It has been hypothesized that treatment with HDACi alters gene expression to favor a proapoptotic response [85]. Gene expression profiling of tumor cells treated with HDACi showed that proapoptotic genes involved in the extrinsic or death receptor pathway [such as Fas or tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)] or the intrinsic or mitochondrial pathway (such as bax and bak) are generally up-regulated, whereas prosurvival genes such as *XIAP* and *bcl-2* are generally down-regulated. The precise roles of the extrinsic versus (vs.) intrinsic pathways in HDACi-induced apoptosis in tumor cells and the mechanisms by which they function remain to be discerned.

The production of reactive oxygen species (ROS) appears to play a role in HDACi-induced apoptosis as well. HDACi treatment may activate the production of ROS. Alternatively, expression of thioredoxin protein 2 (TBP2), a ROS regulatory protein that is expressed at low levels in many human cancers, is selectively increased by vorinostat. TBP2 may perhaps bind and inactivate thioredoxin, rendering cells more susceptible to oxidative stress [106]. ROS scavengers have been shown to suppress apoptosis after treatment with HDACi. Hyperacetylation of non-histone proteins may trigger unique apoptotic mechanisms. HDACi acetylate and destabilize Hsp90, a non-histone chaperone protein for a variety of oncoproteins, including Akt and c-raf, resulting in degradation of these oncoproteins [107]. Ku70 is a DNA end-joining protein that sequesters the apoptotic protein Bax in the cytoplasm. When acetylated after treatment with HDACi, Ku70 releases Bax to the mitochondria to induce apoptosis [108, 109]. The HDAC6-specific inhibitor tubacin has been shown to cause accumulation of acetylated α-tubulin but not histones. Additionally, tubacin in certain cell

contexts does not inhibit cell progression but rather may elicit antitumor effects by affecting migration or aggresome function [110].

1.8.3 Antiangiogenic effects of HDACi

The HDACi have recently been shown to be potent angiostatic agents *in vitro* and *in vivo*. In both endothelial and tumor cells, HDACi have been shown to down-regulate angiogenesis-related gene expression. Within cancer cells, HDAC gene expression is thought to be transcriptionally activated by hypoxia, resulting in the down-regulation of tumor suppressor genes p53 and von Hippel-Lindau. HDACs complex with hypoxia-inducible factor 1α (HIF1α) under hypoxic conditions. This leads to increased HIF1α transcriptional activity and increased expression of HIF1α target genes, such as vascular endothelial growth factor (VEGF) [101, 111, 112]. HDACi have been shown to up-regulate inhibitors of angiogenesis, such as p53 and von Hippel-Lindau, and down-regulate angiogenesis-promoting factors, such as VEGF and HIF1α, as well as platelet-derived growth factor and basic fibroblast growth factor. Furthermore, HDACi down-regulate gene expression of survivin, an apoptotic inhibitor, in endothelial cells alone. It therefore appears that the antiangiogenic effects of HDACi may be exerted through inhibition of tumor endothelial cell growth as well as through altering the expression of genes to produce antiangiogenic effects [101, 111, 112]. The antiangiogenic effects of TSA, MS 275 and VPA were also linked to a decrease in the generation of nitric oxide (NO) by endothelial cells and a marked reduction in the expression of the endothelial nitric-oxide synthase (eNOS) [100, 111].

1.8.4 HDACi effects in animal studies

HDACi have been tested in several animal models including human breast, prostate, lung, pancreatic, ovarian, and stomach cancer, melanoma, synovial sarcoma, osteosarcoma, NB,

medulloblastoma multiple myeloma, and leukemia. Human lymphoma SCID mouse models and transgenic mouse models of acute promyelocytic leukemia have also been tested. Overall, inhibition of tumor growth was observed with little toxicity. Agents that have been or are being currently investigated in phase I and phase II clinical trials include TSA, depsipeptide, MS 275, FK 228, and PXD101. The accumulation of acetylated histones caused by HDACi in tumor and normal tissues such as spleen and peripheral blood mononuclear cells (PBMCs) further suggests that this may be an important biomarker to measure in clinical trials [113]. Vorinostat has also been shown to cross the blood-brain barrier in an R6/2HD mouse model and increase histone acetylation in the brain [114]. TSA, vorinostat, VPA, phenylbutyrate, and depsipeptide have been shown to block angiogenesis *in vivo*. Anti-proliferative effects may thus be exerted through the induction of apoptosis and the interference with tumor's establishment of adequate vascularization [98].

1.9 Clinical trials with HDACi

Several HDACi have been investigated in phase I and phase II clinical trials, as single agents and in combination with cytotoxic therapies (Table 1.4). SAHA (Vorinostat) and VPA are the most studied HDACi.

Class	**Compound**	**Phase of Development**	**Potency (cells)**	**HDAC target[a]**
Hydroxamate	Vorinostat (SAHA)	approved	μM	Classes I, II
	PXD101	I/II	μM	Classes I, II
	ITF2357	I	nM	Classes I, II

Cyclic peptide	Depsipeptide (FK228)	I/II	nM	HDAC1, -2
Aliphatic acid	VPA	I/II	mM	Classes I, IIa
	Phenylbutyrate	I/II	mM	Classes I, IIa
Benzamide	MS 275	I/II	μM	HDAC1, -2, -3

Table 1.4: Examples of HDACi in clinical trials. [a] Based on relative sensitivity to HDACi (adapted from references 78 and 98)

1.9.1 SAHA (Vorinostat)

SAHA is a hydroxamic acid inhibitor of HDACs. It has been shown to inhibit the activity of HDACs in both class I and class II but does not inhibit the activity of class III HDACs [81, 115, 116]. SAHA selectivity alters only 2-10% of expressed genes in different transformed cells-with the number of genes whose expression increased about equal to the number whose expression decreased [117]. Although the mechanisms of SAHA activity are not completely understood, it is clear that SAHA induces cell death of transformed cells by selectively altering gene expression and the function of proteins that cause caspase-dependent and caspase-independent cell death [81, 85, 115, 116, 118-122]. It arrests cell growth of a variety of transformed cells in culture at 2.5-5.0 μM [81, 102, 106, 115, 116, 123-125]. Initial trials of intravenous vorinostat in patients with refractory hematologic and advanced solid tumors showed that this agent was well tolerated, with neutropenia and thrombocytopenia being the dose-limiting toxicities in the patients with hematologic malignancies. Vorinostat inhibited HDACs and caused the accumulation of acetylated histones in both normal and malignant

tissues [126]. Subsequently, an oral preparation of vorinostat was tested in phase I trial of 73 patients with advanced solid tumors. The major dose-limiting toxicities observed were anorexia, dehydration, diarrhea, and fatigue. Two of 4 patients with mesothelioma experienced partial responses, and a placebo-controlled, randomized phase III study of oral vorinostat is now open for patients with mesothelioma in whom treatment with pemetrexed has failed [127]. Vorinostat has been partially effective in patients with cutaneous T-cell lymphoma (CTCL). In a phase II trial of oral vorinostat in patients with CTCL, 8 of a total of 33 patients enrolled in the study achieved a partial response, and 14 patients received relief from pruritus. The most common grade 3/4 drug-related adverse events were thrombocytopenia and dehydration [128]. On October 6, 2006, the U.S. Food and Drug Administration granted approval to vorinostat (Zolinza) for the treatment of CTCL in patients with progressive, persistent, or recurrent disease on or after 2 systemic therapies.

1.9.2 VPA

VPA has been used an an anticonvulsant for decades. It suppresses generalized and partial seizures, acts as a mood stabilizer, and augments the treatment of migraine and neuropathic pain [129]. Recently, cancer treatment has become a new sphere of application for VPA. VPA affects the growth and differentiation of a variety of malignant cells *in vitro* [130]. Similar to teratogenicity, the antitumor effect of VPA is independent of the drug's antiepileptic activity because VPA can be modified to exhibit one effect or the other [131]. Antineoplastic activity includes an effect on the cell cycle through prolongation of the G1 phase [132]. VPA also has a differentiating effect, for example, as shown in NB cells, in which it causes increased expression of neural cell adhesion molecules (NCAM), decreased expression of n-myc oncoprotein, and morphologic alterations such as neuritogenesis [133-136]. In addition, VPA affects the cytoskeleton [137], inhibiting cell motility and tumor metastasis, and demonstrates

antiangiogenic activity *in vitro* [100]. The antineoplastic and teratogenic effect of VPA was shown to depend mainly on the inhibition of HDAC [135, 138]. VPA inhibits class I HDAC1, -2 and -3, and class II HDAC4, -5 and -7 [139]. VPA is now in clinical trial in patients with acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) [140, 141, 142]. VPA is also used as maintenance therapy for childhood malignant glioma after post-operative combined chemotherapy and irradiation [142, 143].

1.10 HDACi and the immune system

The influence of HDACi on antitumor responses of the innate immune system has been described. The effects were mostly based on priming malignant cells with HDACi for immune cell-mediated killing. Cinatl et al [144] reported that treatment of NB cells with 0.5 mM VPA increased their sensitivity to lymphokine-activated killer lysis. Sodium butyrate (SB) was used to induce the expression of co-stimulatory/adhesion molecule on AML cells, thereby effectively inducing tumor immunity. SB up-regulated CD86 and ICAM-1 expression in several AML cell lines and enhanced allogenic mixed leucocyte reaction against HL60 cells [145]. Cancer cells were also shown to become susceptible to NK cell killing after exposure to HDACi like SAHA and FR901228. This susceptibility was due to glycogen synthase kinase-3-dependent expression of MICA and MICB [146]. The specific priming of malignant cells for innate immune effector mechanisms was again reported to be mediated by VPA [147]. Here the authors showed that VPA increased transcription of MICA and MICB in hepatocellular carcinoma cells, leading to increased cell surface, soluble and total MIC protein expression, as well as increased lysis by NK cells. Furthermore, depsipeptide was shown to up-regulate tumor death receptor TRAIL-R2 (DR5) and thereby potentiate NK cell-mediated tumor killing [148].

HDACi have been reported to comprise a new class of immunosuppressive agents [149]. The investigators showed that HDACi FR901228 inhibited CD4 T-cell proliferation in a manner which was not caused by apoptosis or decreased viabilty. FR901228 also abrogated the characteristic aggregation of T cells following activation without affecting early intracellular signals such as tyrosine kinase activity and elevation of intracellular calcium concentration. This effect was reported to correlate with diminished activation-induced expression of the adhesion molecules, LFA-1 and ICAM-1. Furthermore, HDACi inhibited activation-induced CD25 (IL-2 receptor α-chain) and CD154 (CD40 ligand) but not CD69 expression on CD4 cells [149]. HDAC6-mediated tubulin deacetylation was shown to be involved in CD3 and LFA-1 orientation and in the organization of the immune synapse in T lymphocytes [150]. However, separate studies ruled out major effects of HDAC inhibition on T-cell proliferative and cytotoxic responses and on IFN-γ production [151-153], thus leaving open the possibility that other immune cell types may be mostly affected by HDACi and thereby contribute to the immunomodulatory properties of these drugs. In this context, recent experiments have shown the capacity of a selective HDACi, LAQ824, to specifically modulate gene expression in macrophages and dendritic cells (DC) leading to impaired chemokine production and to preferential stimulation of Th2 versus Th1 T lymphocytes [154]. Also, VPA and MS 275 reportly impaired DC differentiation by preventing the acquisition of the DC hallmark CD1a and by affecting the expression of co-stimulatory (CD80 and CD86) and adhesion (ICAM-1 and DC-specific intercellular adhesion molecule 3-grabbing nonintegrin (DCSign)) molecules [155]. In addition, the authors demonstrated that macrophage inflammatory protein-3β/chemokine, motif CC, ligand 19-induced migration, immunostimulatory capacity, and cytokine secretion by DCs were profoundly impaired. The observed defects in DC function on exposure to HDACi were suggested to reflect the obstruction of signaling through nuclear factor kappa B (NFκB), IFN regulatory factor (IRF)-3, and IRF-8 [155]. Reduction of pro-inflammatory cytokine production was also reported for the HDACi SAHA and ITF2357.

Human PBMCs stimulated with lipopolysaccharide (LPS) in the presence of SAHA [153] or ITF2357 [156] released less tumor necrosis factor (TNF)-α, IL-1-β, IL-12, and IFN-γ. Other studies revealed that administration of HDACi to mice ameliorated the autoimmune manifestations of graft-versus-host disease, systemic lupus erythematosus, concanavalin A-induced hepatitis, experimental autoimmune encephalomyelitis, rheumatoid arthritis, and colitis [151-153, 157-160]. How the immune effects of HDACi are exactly mediated is presently unclear.

1.11 Nucleoside Analogs

Nucleoside analogs represent a novel group of cytotoxic antimetabolites in the treatment of hematological malignancies, solid tumors and viral infections [161-164]. They mimic physiological nucleosides and share their metabolic pathways. Most nucleosides enter cells via specialized plasma membrane nucleoside transporter (NT) and are phosphorylated by cellular kinases to their cytotoxic 5'-triphosphates, which affect RNA and DNA synthesis and other metabolic targets.

1.12 1-β-D-arabinofuranosylcytosine (Cytosine arabinoside, cytarabine, araC)

AraC is one of the most important antileukemic drugs currently available for the treatment of AML [165, 166] and large cell lymphoma [167]. AraC was shown to be active against T-lymphoid H9 cells [168]. Continuous cultivation of T-lymphoid C8166 cells in the presence of araC resulted in significantly decreased expression of the major cellular receptor molecule CD4 and co-receptor molecule CXCR4 of T-lymphotropic HIV-1 isolates [169]. AraC is a deoxynucleoside analog that has to be converted into its active triphosphate derivative (araCTP) to exert its cytotoxic effect [170]. AraCTP is then incorporated into the DNA causing chain termination, resulting in a block in DNA synthesis and facilitating programmed

cell death [171]. AraC is a hydrophilic molecule and as such requires facilitated diffusion via nucleoside-specific membrane transport carriers to enter cells [172, 173]. The human equilibrative nucleoside transporter (hENT1) is responsible for 80% of araC influx in human leukemic blast cells [173, 174]. Inside the cell, conversion of araC into its monophosphate derivative araCMP by deoxycytidine kinase (dCK) is believed to be the rate-limiting step in the metabolism of araC [175, 176]. Subsequently, araCMP is phosphorylated into its diphosphate derivative araCDP by nucleoside monophosphate kinases, which in turn finally is phosphorylated into araCTP by diphosphate kinases [177]. In addition to araC a variety of other deoxynucleoside derivatives are active in both hematological and solid malignancies. The purine analogs 2-chlorodeoxyadenosine and fludarabine are active against indolent lymphoid malignancies and are currently used for the treatment of hairy-cell leukemias and chronic and acute leukemias, respectively [178]. The pyrimidine analog gemcitabine has activity in various solid malignancies and some hematological disorders [179]. Gemcitabine-monophosphate prodrug was also shown to be active against thyroid cancer cells *in vitro* [180]. The thymidine (3'-azido-2',3'-dideoxythymidine, 2',3'-didehydro-3'-deoxythymidine) and deoxycytidine (2',3'-dideoxycytidine, 2',2'-difluoro-2'-deoxycytidine) analogs were shown to be active against T-lymphoid H9 cells [168]. The cytidine analog 5-aza-2'-deoxycytidine is a potent hypomethylating agent and has shown to be active in the treatment of AML, chronic myeloid leukemia (CML), and MDS [181]. These compounds are activated intracellularly via the same metabolic pathway as araC.

1.13 Mechanisms of resistance to araC in transformed cells

Prolonged, *in vitro* and *in vivo*, treatment with araC has resulted in the emergence of drug resistant cells with diminished sensitivity to the drug and ultimately contributing to treatment failures [166, 182, 183]. Mechanisms of resistance to araC that have been reported include increased inactivation of araC by cytidine deaminase, decreased intracellular permeation,

decreased cellular activation by dCK, increased degradation of araC-nucleotides by 5'-nucleotidase, imbalance of cellular deoxynucleotide pools, and increased capability of repair of damaged DNA [182-189].

1.14 Methods for measuring NK cell cytotoxicity

Fast, sensitive and material-sparing methods to measure NK cytolytic activity, an important determinant of NK cell function, are critical for the analysis of physiological functions as well as pathological states of the immune system. The gold standard for measuring cell-mediated cytotoxicity has been the chromium (^{51}Cr) [190, 191] or europium (Eu^{3+}) release assays [191-194]. However, measurement of target cell lysis by ^{51}Cr or Eu^{3+} release assays is a time-consuming method requiring labeling of target cells with toxic or radioactive substances that present also a drastic manipulation. Moreover, the large number of cells required by these methods results in high background values. Several other methods for measuring cell cytotoxicity are inconsistent and have numerous shortcomings (table 1.5) [195]. Corey et al [196] also extensively described the shortcomings of other assays for measuring cytotoxicity: Traditional enzyme release assay like lactate dehydrogenase (LDH) is slow and lack sensitivity. Direct cell counting by dye invasion is tedious and relatively insensitive to small amounts of damage; it also suffers from operator error and subjective judgements. Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assays are generally used for the study of apoptosis as they are specific for DNA fragmentation, but they are labor-intensive and are difficult to interprete. Metabolism-based assays generally involve the addition of a chemical which generates a chromophore when it is metabolically processed- this is effective for determining the remaining percentage of life cells, but is insensitive to small rates of cell death or membrane damage for statistical reasons that are inherent to the method. Due to these reasons, the search for other methods that are non-hazardous, easy to perform, and with high sensitivity are highly required.

Table 1.5: Categories of cytotoxicity assays reported in literature[a]

	1a: Target cell death by substance release	1b: Target cell death by cell visualization	2a: Target cell survival by substance uptake/retention	2b: Target cell survival by cell visualization	3a: Effector activity by substance release	3b: Effector activity by cell visualization
Parameters	^{51}chromium, europium-DTPA, DNA-fragmentation, LDH, KLUK, glyceraldehyde 3-phosphate dehydrogenase, luciferase, beta-galactosidase	PI, 7AAD, annexin V, TO-PRO-iodide, decrease in forward light scatter or antibody binding	MTT, 4-Methyl-umbelliferyl-heptanoate, alamarBlue, gammaglutamyl, transpeptidase	PI, 7AAD exclusion, scatter	granzyme B	conjugate formation
Methods	gamma camera, fluorimetry, ELISA, luminescence, photometry, Elispot	flow cytometry, fluorimetry	colorimetry, fluorimetry, photometry	flow cytometry	ELISA Elispot	flow cytometry
Advantage	all target death detected unless substance reuptake by surviving targets and/or effectors	easy handling, direct visualization, reduced cell manipulation unless stained before coculture	reduced manipulation of cells unless marked before coculture	easy handling, direct visualization	detection of effector molecule of cytotoxic activity	visualization of conjugate formation
Disadvantage	extensive cell manipulation, difficulty with labeling, some radioactivity, cell losses due to washing, in part transduction, target cell purity of >80% required	escape from detection by disintegration of cells, gating difficulty due to change in properties of dead cells	uptake of substance by effectors not easy to quantify, target cells in good condition with sufficient metabolic activity needed, target cell purity of >80% required	dependent on precise sample preparation and measurement	restricted to granule dependent activity	conjugate formation not equal to killing

[a] The categories are defined by approach used to measure the results of an interaction between effector cells and target cells (adapted from reference 195)

1.15 Aim of research

Adequate measurement of cytolytic activity of NK cells is necessary to determine their functional status. This research focuses firstly on the establishment of a highly sensitive, safe, material saving, easy to perform, and reliable method for the measurement of NK cytolytic activity. Two research projects were then investigated using the established method.

NK cells as components of the innate immunity substantially contribute to the elimination of virus-infected cells as well as antitumor immune response [60]. Modulation of immune responses which may be important for NK cell activity through epigenetic mechanisms such as acetylation and deacetylation has been studied by some groups. Most of these studies were based on the effects of HDACi on the expression of co-stimulatory/adhesion molecules (e.g. CD86 and ICAM-1), tumor death receptors like TRAIL-R2 (DR5) and ligands for the activating receptors of NK cells (e.g. MICA, MICB, ULBP 1-3) in leukemic and tumor cell lines respectively (see section 1.10) [145-148]. Other studies were based on the direct effect of HDACi on pro-inflammatory cytokine production in T cells, DCs, and PBMCs (section 1.10) [149-156]. On the other hand, nothing has been reported in literature on the direct effect of HDACi on NK cell cytotoxic activity. The first research studied by using the established method for measuring NK cell cytotoxicity focuses on the direct effect of two HDACi, SAHA and VPA, on the cytolytic activity of NK cells. Since cytotoxic activity of NK cells is a complex process that requires adhesion to target cells, synapse formation, and signal transduction leading to granule polarization and exocytosis, it is imperative to determine the steps effected by HDACi. Towards this end, the effects of SAHA and VPA on (1) the surface expression pattern of adhesion molecule (LFA-1), IL-2 receptors (CD25, CD122, CD132), NK cell triggering receptors (NKp30, NKp44, NKp46, NKG2D, DNAM-1), NK cell inhibitory receptors (NKG2A, KIR) (2) intracellular perforin and Granzyme B expression (3)

activation of NFκB signaling and (4) granule exocytosis and IFN-γ production in NK cells were investigated.

As mentioned in section 1.11, chemotherapy is insufficient in the clearance of hematological malignancies and solid tumors. Although the treatment of leukemia like AML has improved, patients that attained complete remission were shown to relapse from minimal residual disease cells that apparently survived chemotherapy [197], giving rise to a more resistant leukemia. Resistance to chemotherapy therefore remains a major obstacle in the treatment of leukemia and solid tumors.

The second aspect of this research studied by using the established method for measuring NK cell cytotoxicity focuses on NK cell cytotoxicity of drug-resistant leukemic cell lines. For this purpose the expression of ligands of NK cell activating and inhibitory receptors on parental and araC-resistant H9 and Molt-4 cell lines as well as their function in NK cell-mediated cytolytic activity was investigated. The possible mechanism involved in the expression pattern of the ligands was also studied.

2.0 Materials and Methods

2.1 Materials

2.1.1 Chemicals

Product	Manufacturer
Absolute Ethanol	J.T. Baker, Stuttgart, Germany
Acrylamide Gel 30	Roth, Karlsruhe, Germany
Ammoniumpersulphate (APS)	Sigma-Aldrich, St. Louis, USA
Antipain	Sigma-Aldrich, St. Louis, USA
Aprotinin	Sigma-Aldrich, St. Louis, USA
Aqua ad injectabila	Delta Select GmbH, Dreieich, Germany
Aqua bidest.	Milli-Q biocel, Millipore GmbH, Eschborn, Germany
BAY11-7085	Calbiochem, Darmstadt, Germany
β-Mercaptoethanol	Sigma-Aldrich, St. Louis, USA
Biocoll Separating Solution (Ficoll)	Biochrom AG, Berlin, Germany
Bovine Serum Albumin (BSA)	Bovine Serum Albumin, Fraction V, PAA Laboratories, Pasching, Austria
Chloroform	Merck, Darmstadt, Germany
Chymostatin	Sigma-Aldrich, St. Louis, USA
Cytofix/Cytoperm	BD Biosciences, San Jose, USA
Cytosine ß-D-Arabinofuranosylcytosine (Cytarabine; araC)	Sigma-Aldrich, St. Louis, USA
Diethylpyrocarbonate (DEPC) water, molecular biology grade	Eppendorf, Hamburg, Germany

Dimethylformamide	Fisher Scientific, Leicestershire, UK
Dimethylsulphoxide (DMSO)	Sigma-Aldrich, St. Louis, USA
Ethylenediaminetetraacetic acid (EDTA)	Merck, Darmstadt, Germany
FACS Clean	BD Biosciences, San Jose, USA
FACS Flow	BD Biosciences, San Jose, USA
FACS Rinse	BD Biosciences, San Jose, USA
Fetal Bovine Serum (FBS)	Sigma-Aldrich, St. Louis, USA
Glycine	Roth, Karlsruhe, Germany
HCl	AppliChem GmbH, Darmstadt, Germany
Hepes	J.T. Baker, Stuttgart, Germany
Human Serum	Blood Bank of the German Red Cross, Frankfurt, Germany
Incidin Perfect	Ecolab, Duesseldorf, Germany
Incidin PLUS	Ecolab, Duesseldorf, Germany
Iscove's Modified Dulbecco Medium (IMDM)	Biochrom AG, Berlin, Germany
Isopropanol	Riedel-de Haeen, RdH, Laboratory Chemicals, Seelze, Germany
Laemmli	Bio-Rad Laboratories, Munich, germany
Leupeptin	Sigma-Aldrich, St. Louis, USA
L-Glutamin	Sigma Chemical Co., St. Louis, USA
$MgCl_2$ solution pack	Applied Biosystems, Darmstadt, Germany
MTT	Serva Electrophoresis GmbH, Heidelberg, Germany
NaCl	Roth, Karlsruhe, Germany

Non-fat milk	Roth, Karlsruhe, Germany
Okadaic acid	Sigma-Aldrich, St. Louis, USA
Orthovanadate (sodium orthovanadate)	Sigma-Aldrich, St. Louis, USA
Paraformaldehyde	Sigma Chemical Co., St. Louis, USA
PD98059	Merck, Darmstadt, Germany
Penicillin	Gruenethal GmbH, Aachen, Germany
Pepstatin A	Sigma-Aldrich, St. Louis, USA
PermWash	BD Biosciences, San Jose, USA
Phenylmethylsulfonylfluoride (PMSF)	Sigma-Aldrich, St. Louis, USA
Phosphate buffered saline (PBS)	Sigma-Aldrich, St. Louis, USA
Ponceau S solution	Sigma-Aldrich, St. Louis, USA
Prestained protein marker	Biolabs, Ipswich, USA
Propidium iodide (PI) stock solution	Sigma-Aldrich, St. Louis, USA
Reagent A	Bio-Rad laboratories, Munich Gemany
Reagent B	Bio-Rad Laboratories, Munich Gemany
Reagent S	Bio-Rad Laboratories, Munich Gemany
Sodium acetate	Riedel-de Haeen, RdH, Laboratory Chemicals, Seelze, Germany
Sodiumdodecylsulphate (SDS)	AppliChem, Darmstadt, Germany
Sodium hydrogen carbonate ($NaHCO_3$)	Merck, Darmstadt, Germany
Sodium hydroxide (NaOH)	Merck, Darmstadt, Germany
Streptomycin sulphate	Sigma-Aldrich, St. Louis, USA
Suberoylanilide hydroxamic acid (SAHA)	Alexis Biochemicals, Gruenberg, Germany
TEMED (N,N,N',N'-Tetramethylethylenediamine)	Roth, Karlsruhe, Germany

TRI® Reagent	Sigma-Aldrich, St. Louis, USA
Tris	SAFC, Andover, UK
Tris base	Sigma-Aldrich, St. Louis, USA
Triton X-100	Merck, Darmstadt, Germany
Trypsin	Difco, Hamburg, Germany
Trypsin inhibitor	Sigma-Aldrich, St. Louis, USA
Tween 20	AppliChem, Darmstadt, Germany
Valproic acid (VPA) (2-Propyl-Pentanoic acid sodium salt)	Sigma-Aldrich, St. Louis, USA
Vincristine (VCR)	Sigma-Aldrich, St. Louis, USA

2.1.2 Media, Buffer and Solution

2.1.2.1 Media, buffer and cell culture solution

PBS 10x concentrate:

95.5 g dry medium was dissolved in 1000 ml Aqua bidest. The solution was then autoclaved.

PBS 1x concentrate:

50 ml PBS from 10x concentrate was diluted in 450 ml Aqua bidest.

Trypsin solution:

0.2% trypsin and 0.02% EDTA were dissolved in 1000 ml PBS. Aliquots were made and stored at -20°C.

Sodium bicarbonate solution 7.5%:

75 g $NaHCO_3$ were dissolved in 1000 ml Aqua bidest. The resulting solution was sterile filtered and stored at 4°C.

L-Glutamine 0.2 M:

29.2 g L-glutamine were dissolved in 1000 ml Aqua bidest. The resulting solution was sterile filtered and stored at -20°C.

Penicillin/Streptomycin solution:

10^6 IE penicillin and 10 g streptomycin sulphate were dissolved in 1000 ml Aqua bidest. The resulting solution was sterile filtered and stored at -20°C.

IMDM basic medium:

17.26 g IMDM dry medium were dissolved in 1000 ml Aqua bidest and sterile filtered using a 0.22 µm filter. Medium was stored at 4-8°C for a maximum of 4 months.

IMDM culture medium:

1% from Penicillin/Streptomycin solution

2% L-Glutamin 0.2 M

4% from sodium bicarbonate solution

10% FBS or 20% FBS, depending on cell to be cultured

83% or 73% IMDM Basic Medium depending on cell to be cultured

Cytotoxicity assay medium:

1% from Penicillin/Streptomycin solution

2% L-Glutamin 0.2 M

4% from sodium bicarbonate solution

1% heat inactivated FBS

92% IMDM Basic Medium

2.1.2.2 Buffer and solution for fluorescent-activated cell sorting (FACS)

FACS buffer (Wash buffer):

2.5 g BSA were dissolved in 500 ml 1x PBS. The buffer was sterile filtered and stored at room temperature.

Paraformaldehyde fixation solution 1%:

10 g paraformaldehyde were dissolved in a mixture containing 400 ml Aqua bidest and 600 ml 1x PBS. The resulting solution was sterile filtered, portioned and stored at -20°C. Thawed aliquots were stored at 4°C.

2.1.2.3 Buffer for cell separation (MACS buffer):

0.5% BSA and 2 mM EDTA were dissolved in 500 ml 1x PBS. The resulting solution was sterile filtered using a 0.22 µm filter and stored at 4-8°C.

2.1.2.4 Buffer for cDNA (RTA buffer):

4000 ml RTA buffer was prepared by mixing 800 µl $MgCl_2$, 400 µl 10x PCR buffer II, 40 µl DNA polymerization mix (200 µM) and 2760 µl DEPC water together. This volume is enough for 25 cDNA dilutions. Storage 4-8°C.

2.1.3 Antibodies and cytokines

2.1.3.1 Monoclonal antibody

Antiboby	**Clone**	**Manufacturer**
AffiniPure F(ab')$_2$ fragment goat anti-mouse IgG, F(ab')$_2$ fragment specific		Jackson ImmunoResearch Laboratories West Grove, USA)

Allophycocyanin (APC)-conjugated mouse anti-human CD3	SK7	BD Biosciences, San Jose, USA
Anti-human MICA/MICB	BAMO1	Immatics Biotechnologies, Tuebingen, Germany
Anti-human ULBP-1	170818	R&D Systems, Wiesbaden, Germany
Anti-human ULBP-2	165903	R&D Systems, Wiesbaden, Germany
Anti-human ULBP-3	166510	R&D Systems, Wiesbaden, Germany
Anti-mouse IgG peroxidase conjugated		Calbiochem, San Diego, USA
Anti-rabbit IgG peroxidase conjugated		Calbiochem, San Diego, USA
Fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD11a/LFA-1	G43-25B	BD Biosciences, San Jose, USA
FITC-conjugated mouse anti-human HLA Class 1	W6/32	Biosource, Camarillo, USA
FITC-conjugated isotype specific goat anti mouse		BD Biosciences, San Jose, USA
FITC-conjugated isotype specific isotype control		BD Biosciences, San Jose, USA
Mouse monoclonal to polio	D171	Abcam, Cambridge, UK

virus receptor (PVR)		
Phycoerythrin (PE)-conjugated anti-human KIR/CD158	180704	R&D Systems, Wiesbaden, Germany
PE-conjugated anti-human CD 132/common γ chain	31134	R&D Systems, Wiesbaden, Germany
PE-conjugated anti-human IL-2 Rβ (CD122)	27302	R&D Systems, Wiesbaden, Germany
PE-conjugated anti-human NCAM/CD56	301040	R&D Systems, Wiesbaden, Germany
PE-conjugated anti-human IL-2 Rα (CD25)	24212	R&D Systems, Wiesbaden, Germany
PE-conjugated isotype specific goat $F(ab')_2$ anti-mouse IgG		R&D Systems, Wiesbaden, Germany
PE-conjugated isotype specific isotype control		R&D Systems, Wiesbaden, Germany
PE-conjugated granzyme B	GB11	Abcam, Cambridge, UK
PE-conjugated Perforin	δG9	BD Biosciences, San Jose, USA
PE-conjugated anti-human NKp30	Z25	Beckman Coulter, Marseille, France
PE-conjugated anti-human NKp44	Z231	Beckman Coulter, Marseille, France
PE-conjugated anti-human	BAB281	Beckman Coulter, Marseille,

NKp46		France
PE-conjugated mouse anti-human NKG2A	Z199	Beckman Coulter, Marseille, France
Purified human NKp30	Z25	Beckman Coulter, Marseille, France
Purified human NKp44	Z231	Beckman Coulter, Marseille, France
Purified human NKp46	BAB281	Beckman Coulter, Marseille, France
Purified human NKG2D	1D11	BD Biosciences, San Jose, USA
Purified mouse anti-human CD226 (DNAM-1)	DX11	BD Biosciences, San Jose, USA

2.1.3.2 Antibodies for Western Blot

Akt antibody (rabbit polyclonal IgG)	Cell Signaling, Beverly, USA
Anti-β-actin, clone AC-15, mouse monoclonal IgG	Sigma-Aldrich, St. Louis, USA
Goat anti-mouse IgG, H & L chain specific (peroxidase conjugate)	Merck, Darmstadt, Germany
Goat anti-rabbit IgG, H & L chain specific (peroxidase conjugate)	Merck, Darmstadt, Germany
Phospho-Akt (Ser473) (193H12; rabbit monoclonal IgG)	Cell Signaling, Beverly, USA
Phospho-p44/42 (pERK1/2) MAP Kinase	Cell Signaling, Beverly, USA

(Thr202/Tyr204) Antibody (rabbit polyclonal IgG)	
p44/42 MAP (ERK1/2) Kinase Antibody (rabbit polyclonal IgG)	Cell Signaling, Beverly, USA

2.1.3.3 Cytokines

Recombinant human IL-2	Cell concept, Umkirch, Germany

2.1.4 Reagents for SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

2.1.4.1 Resolving gel composition (for 2 gels)

12 ml	10%
H_2O	3.5 ml
30% Acrylamide	4.0 ml
1 M Tris/HCl pH 8.8	4.5 ml
10% SDS	120 µl
10% APS	90 µl
TEMED	15 µl

2.1.4.2 Stacking gel composition (for 2 gels)

3.5 ml	5%
H_2O	2.48 ml
30% Acrylamide	0.55 ml
1 M Tris/HCl pH 6.8	0.44 ml
10% SDS	35 µl
10% APS	24 µl

TEMED	4.5 µl

2.1.4.3 Protease inhibitor mix (PIM)

15 mM Antipain pH 7.4	1 mg Antipain dissolved in 1 ml Hepes
15 mM Aprotinin pH 7.4	1 mg Aprotinin dissolved in 1 ml Hepes
15 mM Chymostatin pH 7.4	1 mg Chymostatin dissolved in 200 µl DMSO + 800 µl Hepes
15 mM Leupeptin pH 7.4	1 mg Leupeptin dissolved in 200 µl DMSO + 800 µl Hepes
15 mM Pepstatin A pH 7.4	1 mg Pepstatin A dissolved in 200 µl DMSO + 800 µl Hepes
15 mM Trypsin inhibitor pH 7.4	1 mg Trypsin inhibitor dissolved in 1 ml Hepes

2.1.4.4 Electrophoresis and transfer buffers

Electrophoresis Buffer (10x), pH 8.3	0.25 M Tris Base
In 1000 ml aqua bidest., stored at 4°C	1.9 M Glycine
	1% SDS
Transfer Buffer (10x), pH 8.3	0.25 M Tris Base
In 1000 ml aqua bidest., stored at 4°C	1.9 M Glycine
	20% Methanol

2.1.4.5 Wash and blocking buffers

Wash Buffer (10x), pH 7.5	500 mM Tris/Cl

In 1000 ml aqua bidest., stored at Room temperature	1.5 M NaCl
	0.3% Tween 20
Blocking Buffer (1x)	50 ml from 1 M Tris/Cl (pH 7.5)
In 1000 ml aqua bidest., stored at Room temperature	200 mM NaCl
	0.05% Tween 20
	3% BSA (or 5% non-fat milk)

2.1.5 PCR

2.1.5.1 Reagents for reverse transcription polymerase chain reaction (RT-PCR)

MuLV Reverse Transcriptase N808-008	Applied Biosystems, Licoln, USA
RNase Inhibitor N808-0119	Applied Biosystems, Licoln, USA
Absolute TM QPCR SYBR Green ROX (500 nM) Mix AB-1163/a (PCR)	ABgene, Hamburg, Germany
DNA-Polymerization Mix 20 mM/dNTP (RT-PCR)	Pharmacia, Ratingen, Germany
$MgCl_2$ Solution 25 mM N808-0010 (RT-PCR)	Applied Biosystems, Licoln, USA
Random Hexamers 50 µM N808-0127 (RT-PCR)	Applied Biosystems, Licoln, USA
10x PCR Buffer II N808-0010 (RT-PCR)	Applied Biosystems, Licoln, USA
QuantiTect Probe PCR Kit (1000) 204345	QIAGEN, Hilden, Germany

(PCR)	
QuantiTect SYBR Green PCR Kit (1000) 204145 (PCR)	QIAGEN, Hilden, Germany

All enzymes were stored at -20°C

2.1.5.2 Primers

Assay Name	Primer design	Forward Primer	Reverse Primer
β-actin	In-house	5'-cgc gag aag atg acc cag at-3'	5'-cag agg cgt aca ggg ata gca-3'
NKp30	Castriconi et al. 2003 [198]	5'-tga tca tgg tcc atc cag ga-3'	5'-aat ggc cag tct ccc ttg g-3'
NKp46	Castriconi et al. 2003 [198]	5'-ggc aga atc tga gcg atg tct t-3'	5'-gct ttt cct ttg gaa cca tga a-3'
ULBP-2	Borchers et al. 2006 [199]	5'-ccc tgg gga aga aac taa atg tc-3'	5'-act gaa ctg cca aga tcc act gct-3'
ULBP-3	Borchers et al. 2006 [199]	5'-aga tgc ctg ggg aaa aca act g-3'	gta tcc atc ggc ttc aca ctc aca-3'

2.1.6 Commercial Kits

Human IFN-γ ELISA	R&D Systems, Wiesbaden, Germany
Human Perforin ELISA	Diaclone Research, Besancon Cedex, France
Human Granzyme B ELISA	Diaclone Research, Besancon Cedex, France
TransAM^{TM} NFκB (p50 and p65)	Active Motif, Carlsbad, USA
Nuclear extract kit	Active Motif, Carlsbad, USA

aCella-Tox™ bioluminescence cytotoxicity assay kit	Cell Technology, Mountain View, USA
MACS human NK cell isolation kit II	Miltenyi Biotec, Bergisch Gladbach, Germany

2.1.7 Diverse materials

Tip One Filter Tips, 0.1-10 µl, 1-100 µl, 1-200 µl, 100-1000 µl	Starlab GmbH, Ahrensburg, Germany
5 ml Polystyrene Round Bottom Tube	Becton Dickinson Bioscience Discovery Labware, Bredford, USA
Costar Stripette 5 ml, 10 ml, 25 ml, 50 ml	Corning incorporated Corning, New York, USA
Microtubes 0.5 ml, 1.5 ml	Sarstedt, Nuernbrecht, Germany
Combitips plus 1 ml, 5 ml, 10 ml, 50 ml	Eppendorf, Hamburg, Germany
1.8 ml, 5 ml, Nunc Cryotube™Vials	Nunc Brand Products Nalge Nunc International, Roskilde, Denmark
2 ml Pipette	Falcon BD Labware, NY, USA
TipOne Extended Length, Natural Tips 1-10 µl, 1-200 µl, 100-1000 µl	Starlab GmbH, Ahrensburg, Germany
Culture flasks, different sizes with filter	Nunc Brand Products Nalge Nunc International, Roskilde, Denmark
Culture flasks, different sizes without filter	Greiner bio-one, Frickenhausen, Germany, Falcon BD Labware, NY, USA
96-well plate transparent	Greiner bio-one, Friekenhausen, Germany

96-well plate white bottom	Greiner bio-one, Frickenhausen, Germany
6-,12-, 24-well plate	Falcon BD Labware, NY, USA
Falcon Tubes, 15 ml, 50 ml	Greiner bio-one, Frickenhausen, Germany
Hand gloves, Peha-Soft Powder free	Paul Hartmann AG, Heidenheim, Germany
Nitrile hand gloves NOBA Glove®Nitril	NOBA Verbandmittel GmbH u. CoKG, Wetter, Germany
Safe Lock Tubes 1.5 ml	Eppendorf, Hamburg, Germany
Microamp 96-well Rention Plate, optical plate P7N 4306737	Applied Biosystems, Foster City, USA
Microamp Optical Adhesive Film	Applied Biosystems, Foster City, USA
Scalpel	Feather Safety Razor CO. LTD, Osaka, Japan
Safeseal Tips	Biozym Diagnostic GmbH, Hess.Oldenburg, Germany

2.1.8 Laboratory equipments and software

Adobe PhotoShop CS2	Adobe Systems incorporated, San Jose, USA
Analysis scale	Sartorius GmbH, Goettingen, Germany
Autoclave	Systec GmbH Labortechnik, Wettenberg, Germany
Camera CC12	Soft Imaging Systems
Centrifuges	Hettich Zentrifugen, Tuttlingen, Germany; Eppendorf, Hamburg, Germany
Cooling Chamber	Viesmann, Germany

CO_2 incubator	Binder, Tuttlingen, Germany
Counter	Carl Roth GmbH & Co., Karlsruhe, Germany
Counting chamber	W. Schreck, Hofheim, Germany
Cell Quest^{Pro}	Becton Dickinson, San Jose, USA
Counter	Carl Roth GmbH & Co., Karlsruhe, Germany
Cover slide	Superior, Lauda-Koenigshofen, Germany
FACSCalibur^{TM}	Becton Dickinson, San Jose, USA
Glass Flasks- different sizes	Schott, Mainz, Germany
Glomax 96 Microplate Luminometer	Turner BioSystems, California, USA
Ice Machine	Scotsman, Milan, Italy
Incubator without CO_2	Heraeus, Hanau, Germany
Magnet Midi MACS	Miltenyi Biotec, Bergisch-Gladbach, Germany
Magnet stirer	GLW Gesellschaft für Laborbedarf GmbH, Wuerzburg, Germany
Microscope IX71/CKX41	Olympus, Hamburg, Germany; Carl Zeiss, Goettingen, Germany
Microsoft Office 2007	Microsoft Corporation, Redmond, USA
Millipore	Millipore, Eschborn, Germany
Mini-PROTEAN® II Electrophoresis Cell	Bio-Rad Laboratories, Munich, Germany
Multi Magnet Stand	Miltenyi Biotec, Bergisch-Gladbach, Germany
Multicanal pipette	Eppendorf, Hamburg, Germany
Multipipette	Eppendorf, Hamburg, Germany
Nitrocellulose membrane	Schleicher & Schuell, Dassel, Germany

7900HT Fast Real-Time PCR System with standard 96-well block module	Applied Biosystems, Licoln, USA
pH-Meter	WTW GmbH, Germany
Photospectrometer GENios Plus	TECAN Deutschland GmbH, Crailsheim, Germany
Photospectrometer Gene Quant II	Pharmacia Biotech, Amersham Biosciences, Freiburg, Germany
Pipetteboy	IBS Integra Bioscience Pipetboy acu, Fernbach, Germany
Pipettes 0.5-10 µl, 2-20 µl, 10-100 µl, 50-200 µl, 100-1000 µl	Eppendorf, Hamburg, Germany ; BioHit Deutschland GmbH ; Gilson, Middleton, USA
Reference Manager® 10	Thomson Research Soft., Carlsbad USA
Refrigerators and Freezers	Liebherr-Holding GmbH, Bieberach, Germany; Bosch, Stuttgart, Germany
Scale	Mettler GmbH, Giessen, Germany
Sterile flow	Heraeus, Hanau, Germany; Kendro, Vienna, Austria; NUAIRE, Plymouth, USA; Microflow Biological Safety Cabinett
Thermomixer	Eppendorf, Hamburg, Germany
Vortexer	Heidolph Instruments, Schabach, Germany

2.1.9 Target cell lines

2.1.9.1 Cell lines purchased from cell bank

Cell line	Cell type	Origin	Cell number	Culture medium
P-815	Mouse mastocytoma	Established from the mastocytoma tumor of a DBA/2 mouse treated with methylcolanthrene	DSMZ: ACC 1	IMDM with 10% FBS+supplements
Jurkat, clone E6-1	Human acute T cell leukemia	The line was cloned from Jurkat-FHCRC cells	ATCC No. TIB-152	IMDM with 10% FBS+supplements
HL-60	Human Promyelocytic leukemia	Established from peripheral blood leucocytes by leukopheresis from a 36-year-old Caucasian female with acute promyelocytic leukemia.	ATCC No. CCL-240	IMDM with 10% FBS+supplements
K562	Human erythroleukemia	Established from the pleural effusion of a 53-year-old female with myelogenous leukemia in terminal blast crises.	ATCC No. CCL-243	IMDM with 20% FBS+supplements
H9	Human T-cell lymphoma	H9 is a clonal derivative of HuT 78, a human T-cell	ATCC No. HTB-176	IMDM with 10%

		line derived from peripheral blood of a patient with sezary syndrome		FBS+supplements
Molt-4	Human acute lymphoblastic leukemia	Molt-4 is a suspension culture derived from the peripheral blood of a 19-year-old male with acute lymphoblastic leukemia in relapse.	ATCC No. CRL 1582	IMDM with 10% FBS+supplements
C8166	Human T-cell leukemia	Derived by fusion of primary umbilical cord blood cells with HTLV-1 producing line from adult T cell leukemia lymphoma patient.	ECACC 88051601	IMDM with 10% FBS+supplements
PC-3	Human Prostate adenocarcinoma	PC3 was initiated from a grade IV prostatic adenocarcinoma from a 62-year-old male Caucasian	ATCC No. CRL 1435	IMDM with 10% FBS+supplements
LNCaP (clone FGC)	Human metastatic prostate adenocarcinoma	This strain was isolated from a needle aspiration biopsy of the left supraclavicular lymph node of a 50-year-old Caucasian male (blood type B+) with	ATCC No. CRL-1740	IMDM with 10% FBS+supplements

		confirmed diagnosis of metastatic prostate carcinoma		
DU145	Human prostate carcinoma, metastasis to brain	DU145 was isolated from a lesion in the brain of a patient with widespread metastatic carcinoma of the prostate and a 3-year history of lymphocytic leukemia	ATCC No. HTB-81	IMDM with 10% FBS+supplements

Buffy coats from healthy volunteer donors were obtained from the blood bank of the German Red Cross Society.

2.1.9.2 Established cell lines

The human MYCN-amplified NB cell lines (UKF-NB-2, UKF-NB-3 and UKF-NB-4) and the alveolar rhabdomyosarcoma cell lines (UKF-Rhb-1 and KFR) were established respectively from bone marrow metastases of patients with diagnosis of NB (stage IV disease) and alveolar rhabdomyosarcoma at the interdisciplinary laboratory for tumor and virus research, institute for medical virology, Johann Wolgang-Goethe University, Frankfurt, Germany [200-205]. The alveolar rhabdomyosarcoma cell line HA-OH1 was a kind gift provided by Dr. E. Koscielniak (Olga Hospital, Stuttgart, Germany). The human rhabdomyosarcoma cell lines (RH-1 and RH-36-embryonal subtypes; RH-28, RH-30, and RH41-alveolar subtypes) were kindly provided by Dr. Peter J. Houghton (St. Jude's Children's Research Hospital, Memphis, TN, USA) [206-208]. The VCR-resistant UKF-NB-2 subline (designated UKF-NB-2^{r}VCR^{10})

and araC-resistant H9 and Molt-4 cell sublines (designated $H9^{r}100^{ARAC}$ and $Molt\text{-}4^{r}100^{ARAC}$ respectively) were established by exposing parental cells to increasing concentrations of the drug [202]. The resistant sublines were grown for more than 6 months in IMDM supplemented with 10% FBS and containing 10 ng/ml VCR or 100 µM araC. Cells were subcultured at 5-day intervals. All experiments were performed using VCR- or araC-resistant cells subcultured at 5-day intervals without further addition of drug for up to 10 passages.

2.2 Methods

2.2.1 Cultivation of adherent eukaryotic cells

RH-1, RH-28, RH-30, RH-36, RH-41, UKF-Rhb-1, KFR, HA-OH1, PC-3, LNCaP, DU145, UKF-NB4, UKF-NB-3, UKF-NB-2 and UKF-NB-$2^{r}VCR^{10}$ cell lines were propagated in IMDM supplemented with 10% FBS at 37°C in a humidified 5% CO_2 incubator. The cells grew into confluent cell race and were subsequently passaged according to the following protocol:

- Old medium is discarded and the cells are washed 2x with sterile PBS.
- 0.2 ml trypsin/EDTA is added to the cells to detach them from culture flask (50 ml, $25cm^2$).
- Cells are incubated at 37°C till they are completely detached (for about 5 min) from flask.
- Detached cells are re-suspended in 10 ml culture medium and distributed into new flasks containing medium according to splitting rate like 1:5, 1:10, 1:20.

2.2.2 Cultivation of eukaryotic suspension cells

Parental and araC-resistant H9 and Molt-4, C8166, P-815, HL-60 and Jurkat cell lines were cultured in IMDM containing 10% FBS at 37°C in a humidified CO_2 incubator. For K562 cell

line, IMDM containing 20% FBS was used for cultivation. Cells were split every 5 days at a splitting rate of 1:10 (2 ml cell suspension + 18 ml culture medium) or 1:20.

2.2.3 Polyclonal NK cell preparation

PBMCs were isolated from the blood of healthy volunteers by Ficoll (Biocoll)-Hypaque centrifugation: Fifteen ml Ficoll was pipetted into a 50 ml falcon tube. Blood was diluted 1.5x, i.e. 15 ml blood was mixed with 10 ml PBS + 2% FBS. The diluted blood was layered on Ficoll without mixing blood with Ficoll. The sample was centrifuged at 4°C for 30 min at 400 g with no brake. After centrifugation, the upper plasma was removed and discarded without disturbing the plasma-Ficoll interface (Fig 2.1). The PBMCs layer at the plasma-Ficoll interface was carefully removed and retained without disturbing erythrocyte/granulocyte pellet.

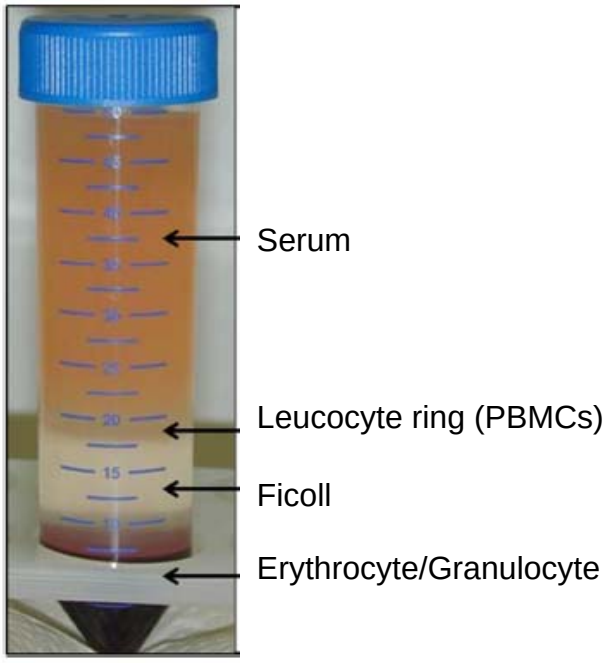


Figure 2.1: Buffy coat separation by density centrifugation using Ficoll

The PBMCs were washed once with PBS + 2% FBS. Freshly isolated PBMCs were transferred into IMDM + 10% human serum and incubated for 2 h at 37°C to allow adherence

of monocytes to the bottom of the 75 cm^2 culture flasks. After incubation, the cell suspension was collected and NK cells were separated according to manufacturer's protocol using the MACS NK cell isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany). Separated NK cells were maintained in IMDM+10% FBS at 37°C in 5% humidified CO_2 incubator. For experiments to determine the direct effect of VPA and SAHA on NK cell cytotoxicity, NK cells were stimulated with 100 U/ml IL-2 and simultaneously treated with VPA or SAHA for 4 days. For other cytotoxic experiments, NK cells were stimulated with or without 100 U/ml IL-2 for 4 days.

2.2.4 Cytotoxicity assay principle

NK cells were tested for cytolytic activity against indicated target cells using the "aCella-Tox" kit (Cell Technology, Mountain View, CA) that employs the coupled luminescent technology for the detection of cytotoxicity (Fig. 2.2) [209]. Target cells were plated in triplicate (5000 cells per well) in a 96 well white plate (Greiner Bio-One, Frickenhausen, Germany). Effector cells (NK cells) at indicated effector to target (E:T) ratios were added. Spontaneous effector- and target cell death was accomplished by including control wells of effector cells at numbers corresponding to those of their various E:T ratios and target cells according to the concentrations used for the assay. Twenty µl of lytic reagent (0.5% NP-40/100 µl sample) was added to the target cells positive control (total glyceraldehyde-3-phosphate dehydrogenase (G3PDH) release) 15 min to end of assay incubation. At the end of incubation, 100 µl of 2x enzyme assay reagent was added to each well. Fifty µl of 1x detection reagent was immediately added to each well. The plate was read at once in a luminometer (Glomax, Turner BioSystems, CA). For the mAb-mediated neutralization experiments, 10 µg/ml each of anti-PVR, anti-ULBP-2, and anti-ULBP-3 mAbs were used to block PVR, ULBP-2 and ULBP-3 expression on target cells. NKG2D was also blocked on NK cells with 10 µg/ml anti-NKG2D Ab. Isotype control IgG was used as negative control.

For redirected killing experiments, 1 µg/ml of purified NKp30 and NKp46 mAbs was used to block NKp30 and NKp46 receptors on NK cells. IMDM supplemented with 1% heat inactivated FBS was used as assay medium. The percent cytotoxicity was calculated as follows: [(experimental G3PDH release - spontaneous G3PDH release from effector cells alone - spontaneous G3PDH release from target cells alone)/(maximum G3PDH release from target cells - spontaneous G3PDH release from target cells)]x100. The spontaneous target cell release was always < 20% of maximum release.

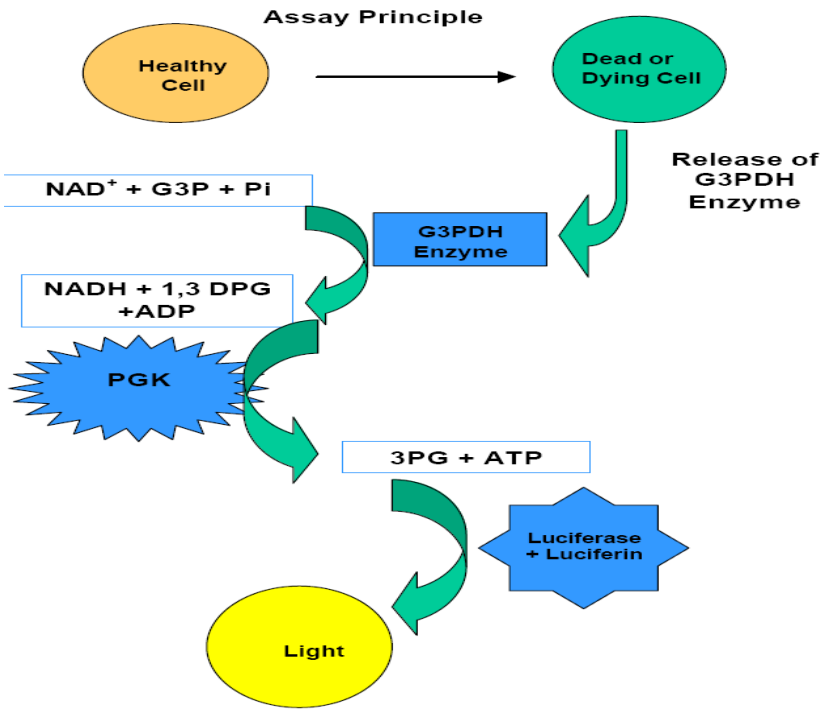


Figure 2.2: assay principle of the coupled luminescent method (adapted from reference 209)

2.2.4.1 Redirected lysis

Redirected lysis is an antibody-mediated lysis of Fc receptor bearing target cells. It is an assay used to determine the specific function of a receptor expressed by cytotoxic cells. To asses the direct effect of VPA and SAHA on the activity of NK cell triggering recptors, NK cells were first cultured for 4 day in IL-2 with or without VPA or SAHA. Five thousand effector cells (both HDACi-treated and control NK cells) were plated in triplicate in a 96 well white plate.

Purified NKp30 and NKp46 mAbs (1 μg/ml) were then used to block NKp30 and NKp46 receptors on effector cells for 30 mins before addition of 5000 target cells. The $Fc\gamma R^+$ P815 cell line was used as target cell line. A 4 h cytotoxicity experiment was performed as described above in section 2.2.4.

2.2.5 The flow cytometric principle

Flow cytometry uses the principles of light scattering, light excitation, and emission of fluorochrome molecules to generate specific multi-parameter data from particles and cells in the size range of 0.5 μm to 40 μm diameter. It is used for the quantification of antigens that are expressed on the cell membrane as well as intracellular antigens. Cells are hydro-dynamically focused in a sheath of PBS before intercepting an optimally focused light source. Lasers (argon laser in the case of FACScan) are most often used as a light source in flow cytometry. As cells or particles of interest intercept the light source they scatter and fluorochromes are excited to a higher energy state. As the molecules relax to a lower state, energy is released as a photon of light with specific spectral properties unique to different fluorochromes (Table 2.1) [210].

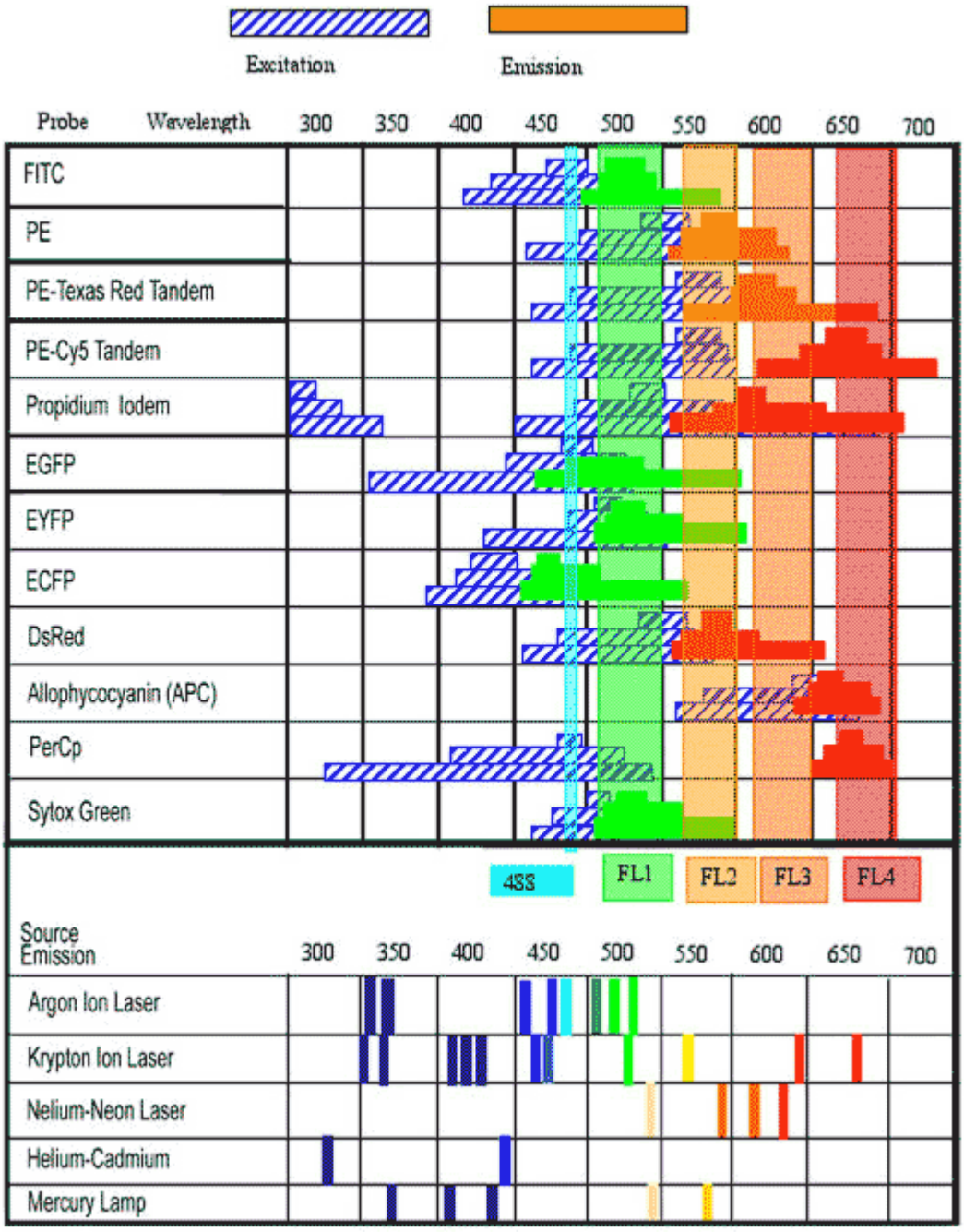


Table 2.1: Fluorescence spectra of commonly used fluorochromes. The bottom part of the table summarizes the emission wavelengths of various light sources used in flow cytometry. The 488 nm line of the argon ion laser is extended over the spectra. (adapted from reference 210, p245).

Photo multiplier tubes (PMT's) are detectors which collect the photon emissions from each "event" and convert them to analog voltages. The electrical pulses originating from light detected by the PMTs are then processed by a series of linear and log amplifiers. Logarithmic

amplification is most often used to measure fluorescence in cells. This type of amplification expands the scale for weak signals and compresses the scale for strong or specific fluorescence signals. After the different signals or pulses are amplified they are processed by an Analog to Digital Converters (ADC's) and recorded as data files (one parameter, two parameter histograms). Optical filters are placed before the detectors so that only wavelengths of light corresponding to specific fluorochrome emissions are collected by each detector (e.g. FITC emits in the green region therefore a 30 nm bandpass filter centered at 525 nm could be used to collect light from this fluorochrome). Light scattered at the same wavelength and direction as the laser light, primarily from the surface of the cell, correlates with relative cell size (Forward Angle Light Scatter (FSC)) while light scattered 90 degrees to the laser (Side Scatter (SSC)) usually from internal structures, correlates with granularity. By correlating these two parameters, one can discriminate subpopulations of cells in peripheral blood samples, for example. Signals corresponding to cell debris or cell aggregates can also be detected and excluded from analysis on the basis of forward and side scatter. One unique feature of flow cytometry is that it measures fluorescence per cell or particle. This contrasts with spectrophotometry in which the percent absorption and transmission of specific wavelengths of light is measured for a bulk volume of sample.

Staining cells with multiple fluorochromes conjugated to antibodies or fluorochromes directed at other specific targets such as DNA, cytokines, or other proteins distinguishes cell subpopulations which can be quantified. Data is displayed and analyzed using histograms or two-dimensional dot plots on a computer system.

Histogram Files: Histogram files can be in the form of one-parameter or two-parameter files and consist of a list of events for a 1 parameter or 2 parameter histogram.

One-Parameter Histograms: A one parameter histogram is a graph of cell count on the y-axis and the measurement parameter on x-axis. All one-parameter histograms have 1,024 channels. These channels correspond to the original voltage generated by a specific light event detected by the PMT detector. In other words, the ADC assigns a channel number based on the pulse height for individual events. Therefore, brighter specific fluorescence events will yield a higher pulse height and thus a higher channel number when displayed as a histogram.

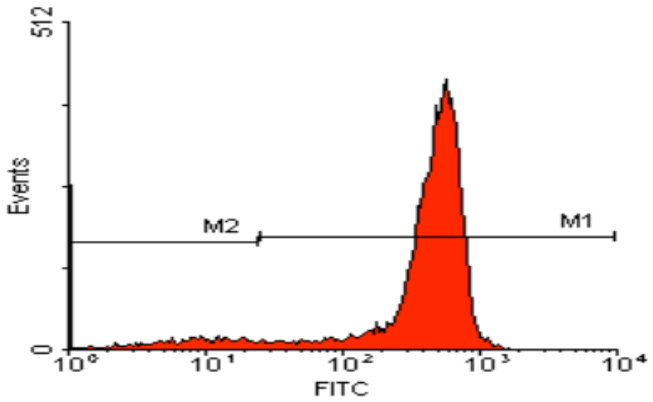


Figure 2.3: one-parameter histogram

Two-Parameter Histograms: A graph representing two measurement parameters, on the x- and y-axes, and cell count height on a density gradient. This is similar to a topographical map. One can select 64 or 256 channels on each axis of two-parameter histograms. Particle counts are shown by dot density or contour plots.

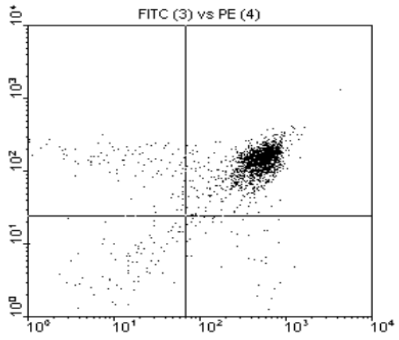


Figure 2.4: two-parameter histogram dot plot displaying FL1-FITC on the x-axis and FL2-PE on the y-axis.

2.2.5.1 Determination of cell cycle using propidium iodide

The cell cycle of a proliferating cell is made up of 4 phases. These phases are characterized by changes in the DNA-content of the cell. The G0/G1-phase (Gap 1) marks the time period after a mitotic division. During this phase, the DNA-content within the cell is diploid (2n). Depending on cell type, the cell may now differentiate or continue to proliferate. Proliferating cells enter into the DNA-synthesis phase (S-phase). At the end of the S-phase, the cells now have a double diploid DNA-content (4n). In the following G2-phase, the DNA-content remains unchanged and the cell synthesizes mitotic active enzymes. Post-replicative repairs may also occur during the G2-phase. Mitosis (M) follows subsequently and the cell divides into two diploid daughter cells which then enter into the G0/G1-phase.
DNA can be stained with DNA-intercalating fluorochrome propidium iodide (PI). Through this means and with the aid of a flow cytometer, the DNA-content as well as the cell cycle profile of a cell population can then be ascertained and graphically represented on a histogram [211].

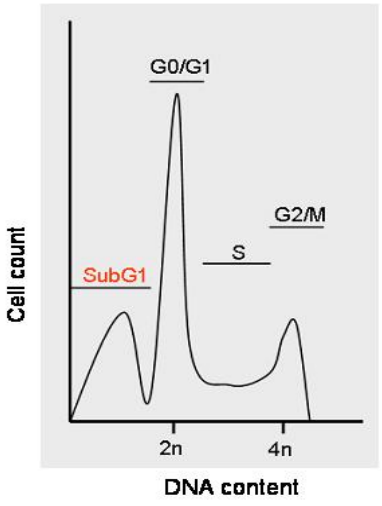


Figure 2.5: Cell cycle distribution- histogram showing sub G1 fraction

The DNA-content of apoptotic cells (sub G1) is less than 2n. This hypodiploidity results from DNA fragmentation during the course of apoptosis and necrosis. However in order to be seen

in the sub G1 area, a cell must have lost enough DNA to appear there; so if cells enter apoptosis from the S- or G2/M-phase of the cell cycle, they may not appear in the sub G1 peak. To detect early apoptotic cells, the cells are first fixed with 70% ethanol. This will permeabilize the membrane, allowing small molecular DNA fragments to be extracted from the cell.

2.2.5.2 Effect of HDACi on NK cell viability

PI staining was used to determine the apoptotic rate in IL-2-activated NK cells upon treatment with HDACi, SAHA and VPA. After 4 days HDACi treatment ($1x10^6$ cells were seeded in a 12 well plate), 2 ml cell suspension was filled into the FACS tubes and centrifuged (5 min, 400 g, 4°C). The supernatant was discarded while the cell pellet was washed with 2 ml PBS and centrifuged. The cells were fixed in 2 ml 70% ethanol for at least 2 h or overnight at -20°C. After fixation, cells were centrifuged, supernatant discarded and cells were washed once with PBS and centrifuged again. The cell pellet was then resuspended in 500 µl PI working solution (100 µl 0.1% Triton X-100 + 200 µl 1 mg/ml PI Stock solution + 9.7 ml PBS). The samples were incubated in the dark for 30 min at room temperature. Afterwards, the cell cycle profile was determined using flow cytometer in FL2-channel (separated in FL2-A and FL2-W, doublet discrimination module). Ten thousand cells were measured from each sample. Untreated IL-2-activated NK cells were used as control. Becton Dickinson FACSCalibur was used for all measurements. Results were evaluated using CellQuest Pro programme.

2.2.5.3 Measurement of cell surface receptors

For quantitative analysis of the expression of cell surface and intracellular receptors, a one color cytofluorometric analysis was carried out. Cells were stained with appropriate mAb as follows:

- Cells were harvested (adherent cells were first trypsinized while suspension cells were distributed directly into polystyrene round bottom 12x75 mm Falcon tubes), washed twice and cell suspension was then adjusted to a concentration of $1x10^6$ cells/ml in FACS buffer.
- Cells were stained with 10-20 µg/ml appropriate mAb according to manufacturer's protocol.
- After incubation, cells were washed twice by centrifugation at 400 g for 5 min and resuspended in 100 µl FACS buffer.
- 20 µl of fluorochrome-labeled secondary Ab were added.
- Cells were incubated at room temperature for at least 20 min in the dark.
- Cells were washed twice, resuspended in 500 µl FACS buffer and analyzed by flow cytometry.
- In case of conjugated mAbs, the secondary Ab step was left out.
- For detection of intracellular antigens (perforin, granzyme B, IFN-β, IFN-γ) cell fixation and permeabilization steps were done prior to staining with mAbs. For this purpose, cells were fixed with 200 µl Cytofix/Cytoperm for 20 min at room temperature, washed and then permeabilized with 200 µl PermWash for 20 min at room temperature.

2.2.6 Principle of RT-PCR

RT-PCR is a laboratory technique for amplifying a defined piece of a ribonucleic acid (RNA) molecule. The RNA strand is first reverse transcribed into its DNA complement or complementary DNA (cDNA), followed by amplification of the resulting DNA using PCR. This can either be a 1 or 2 step process. PCR itself is the process used to amplify specific parts of a DNA molecule, via the temperature-mediated enzyme DNA polymerase. In the first step of RT-PCR, called the "first strand reaction," cDNA is made from a messenger RNA

template using dNTPs and an RNA-dependent DNA polymerase (reverse transcriptase) through the process of reverse transcription. RT-PCR exploits a characteristic of mature mRNAs known as the 3' polyadenylated region, commonly called the poly(A) tail, as a common binding site for poly(T) DNA primers. In the case of bacterial mRNA, which lack a poly(A) tail sequence-specific primers can be generated to amplify the target mRNA sequence. These primers will anneal to the 3' end of every mRNA in the solution, allowing 5'->3' synthesis of cDNA by the reverse transcriptase enzyme. cDNA can also be prepared from mRNA by using gene specific primer or random hexamer primers.

After the reverse transcriptase reaction is complete, and cDNA has been generated from the original single-stranded mRNA, standard PCR, termed the "second strand reaction," is initiated. If the initial mRNA templates were derived from the same tissue, subsequent PCR reactions can be used to probe the cDNA library that was created by reverse transcription. Primers can be designed to amplify target genes being expressed in the source tissue. Quantitative real-time PCR can then be used to compare levels of gene expression.

1. A thermostable DNA polymerase and the upstream and downstream DNA primers are added.
2. The reaction is heated to temperatures above 37°C to facilitate sequence specific binding of DNA primers to the cDNA.
3. Further heating allow the thermostable DNA polymerase to make double-stranded DNA from the primer bound cDNA.
4. The reaction is heated to approximately 95°C to separate the two DNA strands.
5. The reaction is cooled enabling the primers to bind again and the cycle repeats.

After approximately 30 cycles, millions of copies of the sequence of interest are generated. The original RNA template is degraded by RNase H, leaving pure cDNA (plus spare primers).

The exponential amplification via RT-PCR provides for a highly sensitive technique, where a very low copy number of RNA molecules can be detected. RT-PCR is widely used in the diagnosis of genetic diseases and, quantitatively, in the determination of the abundance of specific different RNA molecules within a cell or tissue as a measure of gene expression.

2.2.7 Real-time RT-PCR (SYBR Green Principle)

SYBR Green provides the simplest and most economical format for detecting and quantitating PCR products in real-time reactions. SYBR Green binds double-stranded DNA, and upon excitation emits light. Thus, as a PCR product accumulates, fluorescence increases. The advantages of SYBR Green are that it is inexpensive, easy to use, and sensitive. The disadvantage is that SYBR Green will bind to any double-stranded DNA in the reaction, including primer-dimers and other non-specific reaction products, which results in an overestimation of the target concentration. For single PCR product reactions with well designed primers, SYBR Green can work extremely well, with spurious non-specific background only showing up in very late cycles. Since the dye binds to double-stranded DNA, there is no need to design a probe for any particular target being analyzed.

2.2.7.1 Real-time RT-PCR

Total RNA was extracted from IL-2-activated NK cells either untreated or treated with 0.5 mM VPA or 0.5 μM SAHA and parental as well as araC-resistant H9 and Molt-4 cell lines using TRI reagent (Sigma-Aldrich, St. Louis, USA) according to the manufacturer's protocol. The TRI reagent allows the simultaneous isolation of RNA, DNA and proteins. The reagent contains in addition to phenol, guanidine isothiocyanate solution which lyses the cells, denatures and inactivates the proteins. The DNA fragments later dissolve in the phenol [212, 213]. Reverse transcription was carried out with reagents from Applied Biosystems (Foster

City, USA) according to the manufacturer's instructions. Relative quantification of gene expression was performed with the ABI PRISM 7900HT in real-time RT-PCR using SYBR Green reagents (Applied Biosystems, Darmstadt, Germany) according to a standard thermal profile: denaturation at 95°C for 15 seconds, annealing/extension at 60°C for 60 seconds with 40 repeats. The β-actin housekeeping gene was used to internally standardize the levels of gene expression. Primers used are as indicated in section 2.1.5.2 of materials and methods. All the samples were performed at least in duplicates. Threshold levels and baseline were optimized. Relative quantification was determined with the SDS2.1 software (Applied Biosystems) provided with the ABI PRISM 7900HT (ΔΔCt method). Relative expression of each transcript was obtained by calculating the ΔC_T as the difference between the PCR C_T of the analyzed gene (NKp46, NKp30, ULBP-2 or ULBP-3) and β-actin used as reference. The difference in expression levels between untreated and treated NK cells was calculated by comparing the ΔC_T of untreated NK cells (used as control) to that of samples from VPA or SAHA treated NK cells. The difference in expression levels between parental cell culture and araC-resistant cell cultures was calculated by comparing the ΔC_T of parental H9 and Molt-4 cells (used as control) to that of araC-resistant H9 and Molt-4 cells. The results are presented as fold decrease/increase.

2.2.8 NK receptors cross-linking and perforin/granzyme B granule release

NK cells were stimulated by mAb cross-linking as previously described [214]. After 4 days of culture in IL-2 with or without VPA or SAHA, cells were labeled with 1 µg/ml appropriate mAbs (NKp30, NKp46) for 30 mins at 4°C. After washing, cells were stimulated with 10 µg/ml AffiniPure F(ab')$_2$ Fragment Goat Anti-Mouse IgG (Jackson ImmunoResearch, West Grove, USA) for 5 min at 37°C. The reaction was stopped with ice-cold PBS. After overnight incubation at 37°C, supernatants were collected for analysis and quantification of granule

release by ELISA assay (Perforin/Granzyme B-ELISA kit, Diaclone Research, Besancon Cedex, France) according to manufacturer's instructions.

2.2.9 Measurement of IFN-γ production

Purified NK cells were treated with 100 U/ml IL-2, and either 0.5 μM SAHA or 0.5 mM VPA were added simultaneously for 72 h. NK cells treated with 100 U/ml IL-2 only were used as control. Supernatants were collected and tested for production of IFN-γ. The amounts of IFN-γ were determined using the Quantikine Human IFN-γ ELISA kit (R&D Systems, Wiesbaden, Germany) according to manufacturer's protocol.

2.3.0 Measurement of NFκB activation

NFκB p50 and NFκB p65 activation were determined using the TransAM^{TM} NFκB Chemi kit (Active Motif, Carlsbad, USA). Purified NK cells were treated with 100 U/ml IL-2, and either 0.5 μM SAHA, 0.5 mM VPA, or 1 μM BAY 11-7085 were added simultaneously for 4 days. NK cells treated with 100 U/ml IL-2 only were used as control. Nuclear extracts were then prepared using the nuclear extract kit (Active Motif, Carlsbad, USA). The extracts were used for the NFκB activation assay according to the manufacturer's protocols. A mutated consensus oligonucleotide (should have no effect on NFκB binding) as well as a wild-type consensus oligonucleotide (a competitor for NFκB binding) was used to monitor the specificity of the assay. Twenty pmol/well of each oligonucleotide was used for the assay.

2.3.1 MTT assay

MTT assay is a laboratory test and a standard colorimetric assay (an assay which measures changes in color) for measuring cellular proliferation (cell growth). It can also be used to determine cytotoxicity of potential medicinal agents and other toxic materials.

Yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) is reduced to purple formazan in the mitochondria of living cells. A solubilization solution (usually either dimethyl sulfoxide (DMSO), an acidified ethanol solution, or a solution of the detergent sodium dodecyl sulfate (SDS) in dilute hydrochloric acid) is added to dissolve the insoluble purple formazan product into a colored solution. The absorbance of this colored solution can be quantified by measuring at a certain wavelength by a spectrophotometer. The absorption maximum is dependent on the solvent employed.

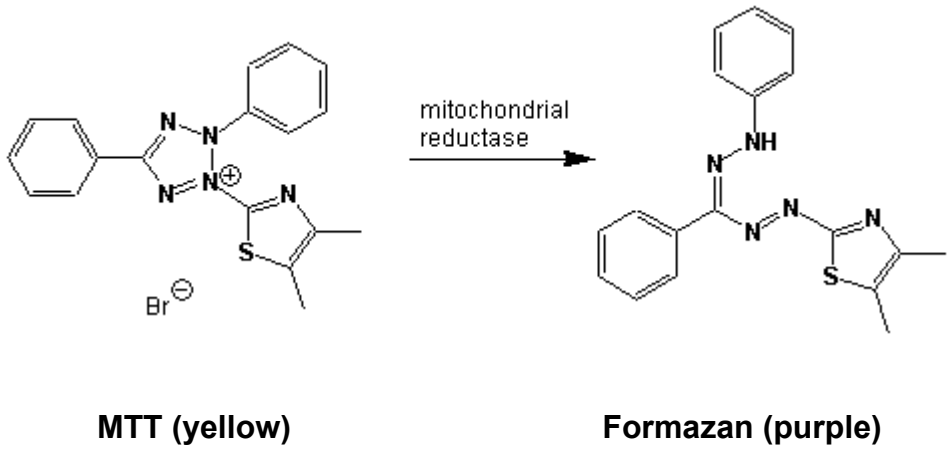


Figure 2.6: MTT reductase scheme

This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion can be directly related to the number of viable (living) cells. When the amount of purple formazan produced by cells treated with an agent is compared with the amount of formazan produced by untreated control cells, the effectiveness of the agent in causing death of cells can be deduced, through the production of a dose-response curve.

2.3.1.1 Cytotoxicity of araC on leukemic cell lines

Cell viability of parental and araC-resistant H9 and Molt-4 cells upon araC treatment was investigated using MTT assay. The cells were grown in 96-well plates with and without

addition of drug. After the incubation period, MTT reagent was added for 4 h. Thereafter, 100 µl of SDS solution (20% SDS in a 1:1 Dimethylformamide (DMF)/H_2O solution) was added for further 4 h. Plates were read on a multi-well scanning spectrophotometer (Tecan, Crailsheim, Germany) at a wavelength of 620 nm and a reference wavelength of 690 nm. Cell viability was determined as the relative reduction of the amount of MTT reduced by cells to its purple formazan derivative, which correlates with the amount of viable cells in relation to cell control. The concentration inhibiting 50% of cell growth (IC_{50}) was calculated by producing a dose-response curve.

2.3.2 Western Blot Principle

A western blot (alternately, immunoblot) is a method to detect a specific protein in a given sample of tissue homogenate or extract. It uses gel electrophoresis to separate native or denatured proteins by the length of the polypeptide (denaturing conditions) or by the 3-D structure of the protein (native/ non-denaturing conditions). The proteins are then transferred to a membrane (typically nitrocellulose or PVDF), where they are probed (detected) using antibodies specific to the target protein. The most common type of gel electrophoresis employs polyacrylamide gels and buffers loaded with SDS. SDS-PAGE (SDS polyacrylamide gel electrophoresis) maintains polypeptides in a denatured state once they have been treated with strong reducing agents to remove secondary and tertiary structure (e.g. S-S disulfide bonds to SH and SH) and thus allows separation of proteins by their molecular weight. Sampled proteins become covered in the negatively charged SDS and move to the positively charged electrode through the acrylamide mesh of the gel. Smaller proteins migrate faster through this mesh and the proteins are thus separated according to size (usually measured in kilo Daltons, kD). The concentration of acrylamide determines the resolution of the gel - the greater the acrylamide concentration the better the resolution of higher molecular weight proteins. Proteins travel only in one dimension along the gel for most blots.

Samples are loaded into wells in the gel. One lane is usually reserved for a marker or ladder, a commercially available mixture of proteins having defined molecular weights, typically stained so as to form visible, colored bands. When voltage is applied along the gel, proteins migrate into it at different speeds. These different rates of advancement (different electrophoretic mobilities) separate into bands within each lane.

It is also possible to use a two-dimensional (2-D) gel which spreads the proteins from a single sample out in two dimensions. Proteins are separated according to isoelectric point (pH at which they have neutral net charge) in the first dimension, and according to their molecular weight in the second dimension.

2.3.2.1 Western Blot analyses of leukemic cells

Parental and araC-resistant H9 and Molt-4 cell lines were washed with ice cold PBS, after which 1 ml freshly prepared Triton X-100 lysis buffer containing 1% orthovanadate, 1% okadaic acid, 1.2% protease inhibitor mix (PIM) and 0.4% PMSF was added. Cells were incubated on ice for 10 min and centrifuged for 7 min at 7500 g. Supernatants containing the cell lysates were collected. Protein concentration of the cell lysates was determined according to BioRad protocols. The same concentration of protein from all samples was diluted 1:1 with Laemmli buffer (950 µl Laemmli + 50 µl β-Mercaptoethanol) and heated for 5 min at 95°C. Samples were stored at -80°C when not used immediately. Samples were subjected to SDS-PAGE (using 10% acrylamide gels) before transfer to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) using the Mini-Protean II system (Bio-Rad, Munich, Germany) according to manufacturer's protocols. After transfer, blots were blocked in blocking buffer containing 3% BSA for 1 h at room temperature to saturate the non-specific protein-binding sites on the nitrocellulose membrane. The following primary antibodies were used: extracellular signal-regulated kinase (ERK), anti-phospho-ERK1/2, AKT, anti-phospho-AKT

(ser 473) (Cell Signaling, Beverly, USA). The blot was incubated overnight with the primary antibody diluted in TBS at 4°C with gentle agitation. Following a 1 h incubation period with peroxidase-conjugated secondary antibody at room temperature visualization was performed by enhanced chemiluminescence (ECL) using a commercially available kit (Amersham, Liverpool, UK).

3.0 Results

3.1 Purity of separated NK cells

NK cells are primarily characterized as CD56 positive and CD3 negative cells. After NK cell separation from PBMCs, the purity of separated NK cells was determined using flow cytometry. The results show 98% of separated cells to be CD56+CD3- (Fig. 3.1).

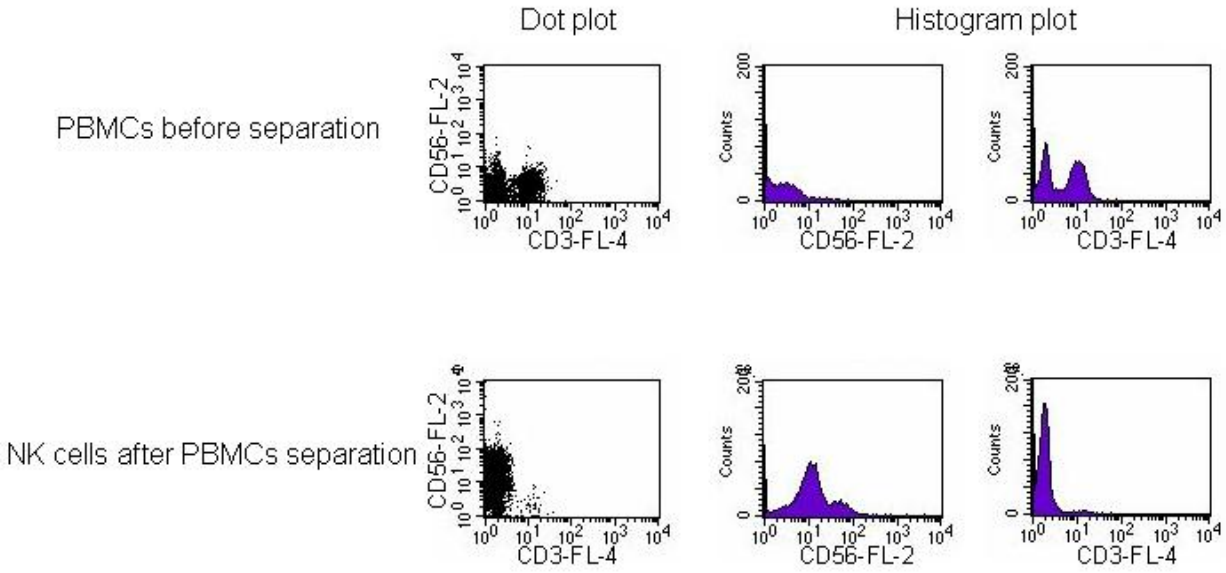


Figure 3.1: Dot- and histogram plots of PBMCs before and after separation with MACS NK cell isolation kit II. Cells were stained with APC-conjugated CD3 and PE-conjugated CD56 mAbs. About 98% of separated cells were CD56+CD3-

3.2 Establishing the coupled luminescent method (CLM) for measuring NK cytotoxicity

We first determined the linear response of "aCella-Tox" within K562 and NB cell lines. This was accomplished by titrating all target cells in the cytotoxicity assay medium from 30,000 to 250 cells/well. Twenty µl of the lytic agent, NP-40, was added to each well. Lysed cells were further incubated for the length of the assay (4 h) before adding the enzyme assay reagent and detection reagent as described in materials and methods. The luminescence was then measured in a luminometer. Up to 20,000 cells/well were within the linear range of the assay (Fig 3.2a). The assay was then standardized using NK sensitive K562 cell line as target and

IL-2-activated NK cell as effector cell. Different E:T ratios, and different incubation times were used. As shown in Fig. 3.2b, K562 target cells were lysed at E:T ratio as low as 0.5:1 (9% ± 2.4% lysis after 2 h). Optimal lysis of target cells was achieved after 4 h of coincubation with IL-2-activated NK cells at an E:T ratio of 4:1 (75% ± 3.13% lysis).

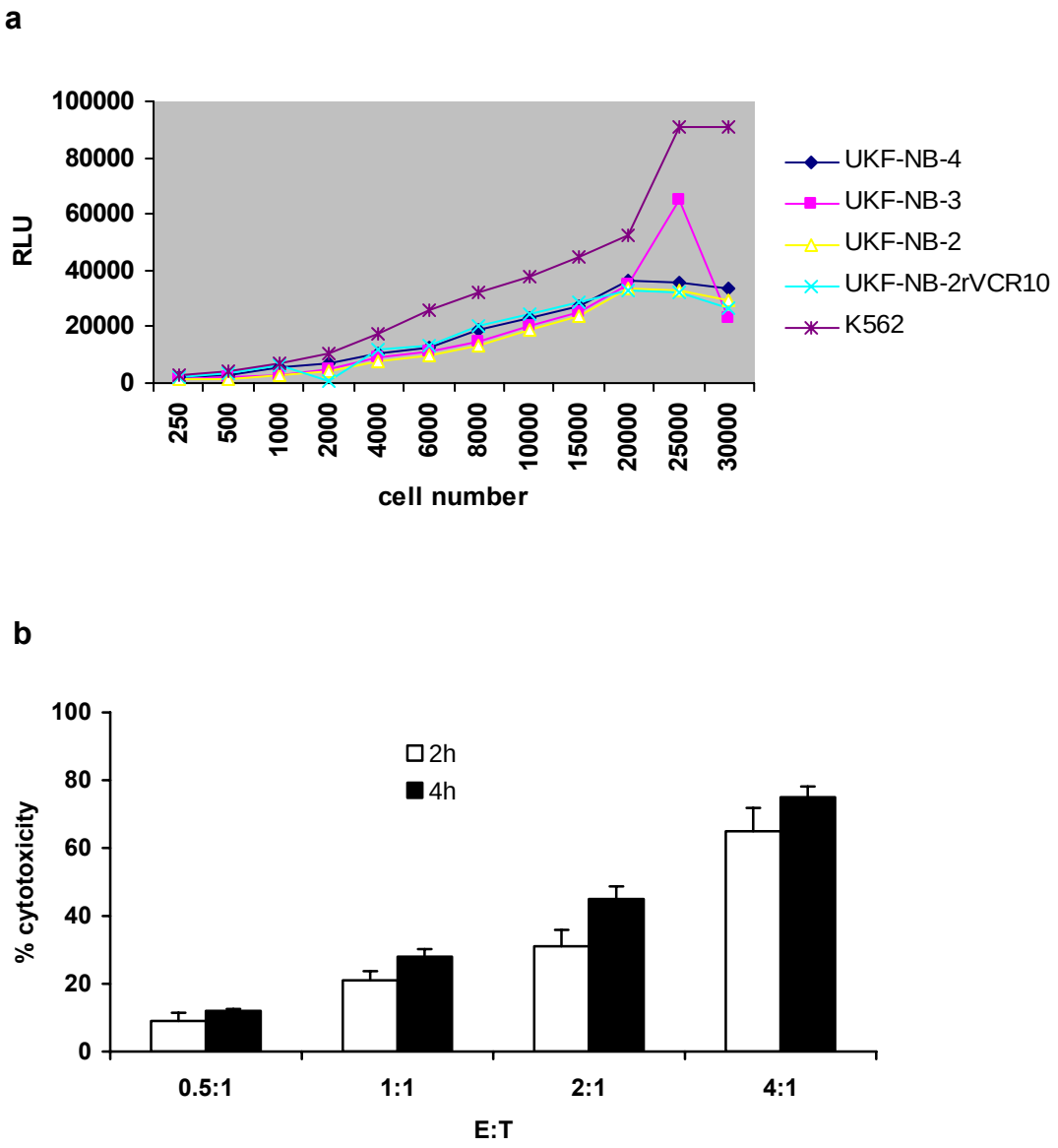


Figure 3.2: [a] Linear response of "aCella-Tox" within K562 and NB cell lines. The relative luminescent unit (RLU) of cells treated with lytic agent for 4 h is shown [b] Cytotoxicity of IL-2-activated NK cells against K562 assessed by G3PDH release**.** Five thousand K562 target cells were coincubated with IL-2 activated NK cells at the indicated E:T ratios for 2 h and 4 h at 37°C. The results are mean ± standard deviation (SD) of 3 independent experiments.

3.3 Validating CLM using NB as target cells

CLM needs to be validated in order for it to be used as a standard method for measuring cytotoxicity. For this purpose, the applicability of CLM was tested by measuring the cytolytic activity of IL-2-activated NK cells against solid tumors (NB cell lines UKF-NB-2, UKF-NB-3 and UKF-NB-4), and chemoresistant NB cell line (UKF-NB-2^{r}VCR^{10}). The results presented in Fig. 3.3 reveal that target cells were lysed in an E:T cell ratio dependent manner. Chemoresistant target cells were generally more sensitive to IL-2-activated NK cell lysis than their respective parental counterparts (45% ± 1% vs. 19% ± 3% respectively for UKF-NB-2^{r}VCR^{10} and UKF-NB-2 at E:T ratio of 4:1).

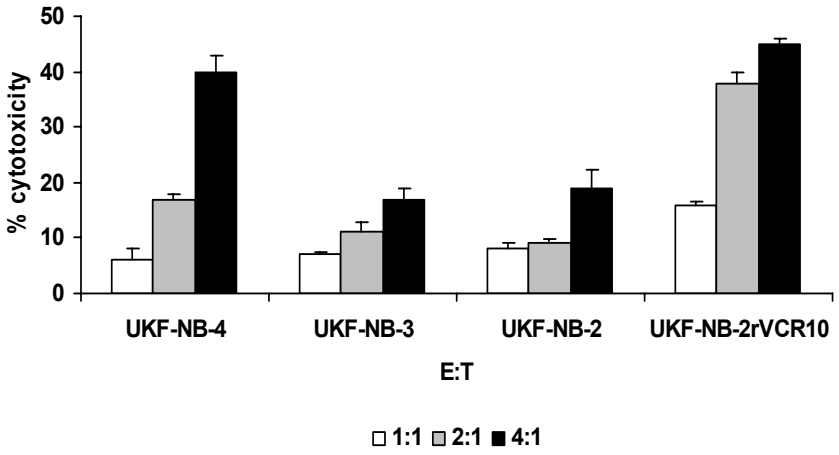


Figure 3.3: IL-2-activated NK cell-mediated cytotoxicity against NB. Killing of parental NB cell lines and VCR-resistant NB cell line was assessed by G3PDH release. Five thousand target cells were coincubated with IL-2-activated NK cells at the indicated E:T ratios for 4 h at 37°C. The results are mean ± SD of 3 independent experiments.

Recently, it was shown that the susceptibility of a subset of NB cells to NK cell-mediated lysis was dependent on the expression level of PVR specifically recognized by DNAM-1 [215-218]. To verify the role of PVR expression in the susceptibility of NB to NK cell lysis, the surface expression of PVR on UKF-NB-2, UKF-NB-3, UKF-NB-4 and UKF-NB-2^{r}VCR^{10} as well as its role in NK cell lysis of NB cell lines were analyzed. PVR was markedly

expressed in all cell lines tested (Fig. 3.4a). Contrasting these results, the expression of other NK activating receptor (NKG2D) ligands like MICA/B were not found on the surface of NB cells. The differential expression of PVR on NB cells seems to correlate with NK sensitivity. Blocking PVR with mAb inhibited NK cell lysis of NB cells (Fig. 3.4b), indicating that NK cell cytotoxicity of NB cells is dependent on the level of PVR expressed on tumor cells.

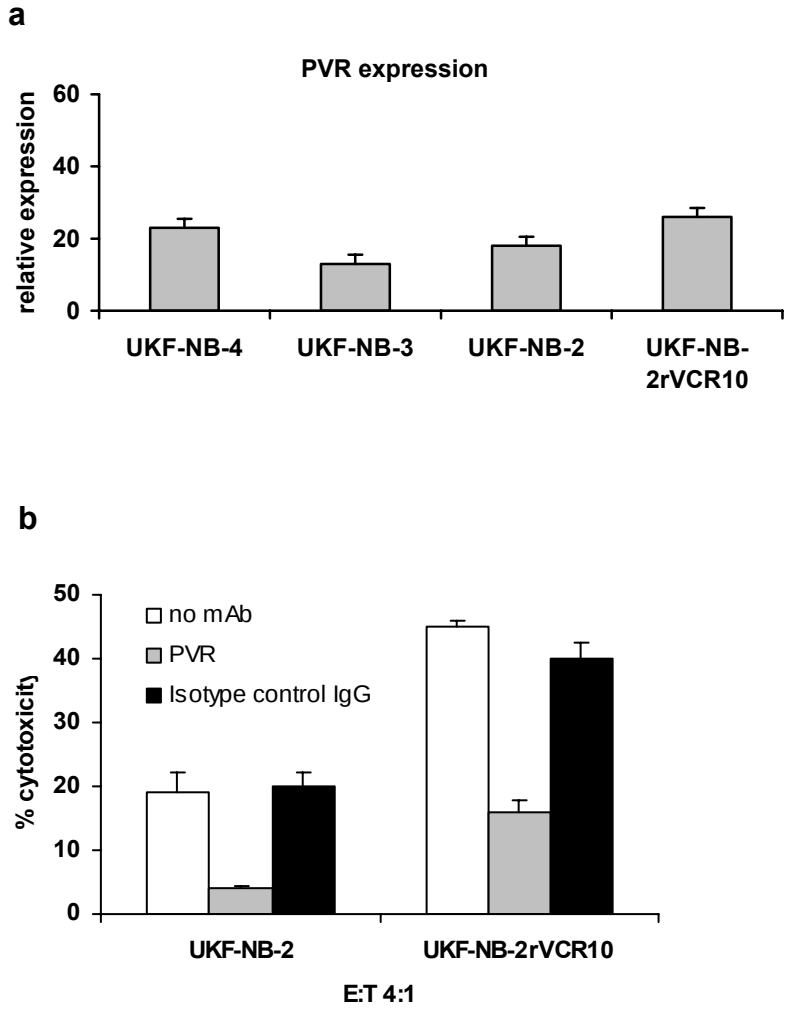


Figure 3.4: Role of PVR in NK cell-mediated cytotoxicity against NB cells. [a] Flow cytometric analysis for the expression of PVR in NB cells. Columns indicate relative expression of one representative of at least five separate experimens. [b] Five thousand NB cells were coincubated with IL-2-activated NK cells at the indicated E:T ratio for 4 h at 37°C either in the absence or presence of 10 µg/ml anti PVR mAb. IgG isotype control was used as negative control. The results are mean ± SD of 3 independent experiments.

3.4 Validating CLM using other cell types as target

CLM was further tested on several other target cell types including rhabdomyosarcoma cell lines (RH-1, RH-28, RH-30, RH-36, RH-41, UKF-Rhb-1, HA-OH1, and KFR), prostate cancer cell lines (LNCaP, DU145, and PC-3), and the human T-cell leukemia cell lines (C8166, H9, and Molt-4). IL-2-activated NK cells were used as effector cells. Rhabdomyosarcoma cell lines were generally resistant to NK cell lysis except for RH-28 and RH-30. The androgen-dependent LNCaP cell line was most sensitive to NK cell lysis while the androgen-independent DU145 and PC-3 cell lines were resistant to NK cell lysis. Of the 3 leukemic cell lines, only H9 and Molt-4 cell lines were sensitive to NK cell lysis (Fig. 3.5).

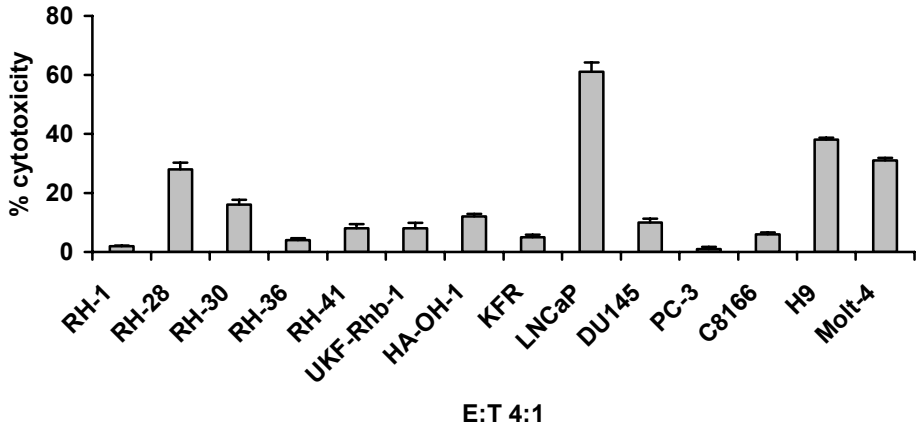


Figure 3.5: IL-2-activated NK cell-mediated cytotoxicity against different cell types. Killing of indicated cell lines was assessed by G3PDH release. Five thousand target cells were coincubated with IL-2-activated NK cells at the indicated E:T ratios for 4 h at 37°C. The results are mean ± SD of 3 independent experiments.

CLM proved to be a highly sensitive method to measure NK cytolytic activity as NK cell lysis can be achieved even at low E:T ratios. The effects of HDACi on NK cell cytolytic activity and the sensitivity of parental and araC-resistant leukemic cell lines to NK cell lysis were thus investigated using CLM.

3.5 Effect of HDACi on viability of NK cells

The effect of HDACi at clinically relevant concentrations [219, 126] (0.25 to 1 mM and 0.5 to 2 μM for VPA and SAHA respectively) on viability of NK cells was first studied. For this purpose, NK cells were cultured simultaneously for 4 days with IL-2 and SAHA or VPA. Dead cells were identified by fractional DNA content ("sub G1 fraction"). NK cells treated with IL-2 alone were used as control. Results revealed SAHA to be clearly toxic to NK cells in the range of therapeutic concentrations [126]. About 48% of NK cells were found in the sub G1-phase (indicating induction of cell death) upon exposure to 2 μM SAHA compared to 9% in control cells (Fig. 3.6). In contrast, 0.5 μM SAHA treatment resulted in no or at most minimal NK cell death, while ≤ 0.5 mM VPA was only slightly toxic to NK cells (1.6% cell death induction when compared to control). Both VPA and SAHA also blocked cell cycle progression into the S- and G2/M-phase in a dose dependent manner (Fig. 3.6). Based on these results, 0.5 mM VPA and 0.5 μM SAHA were selected to investigate the influence of non-toxic HDACi concentrations on NK cell activity.

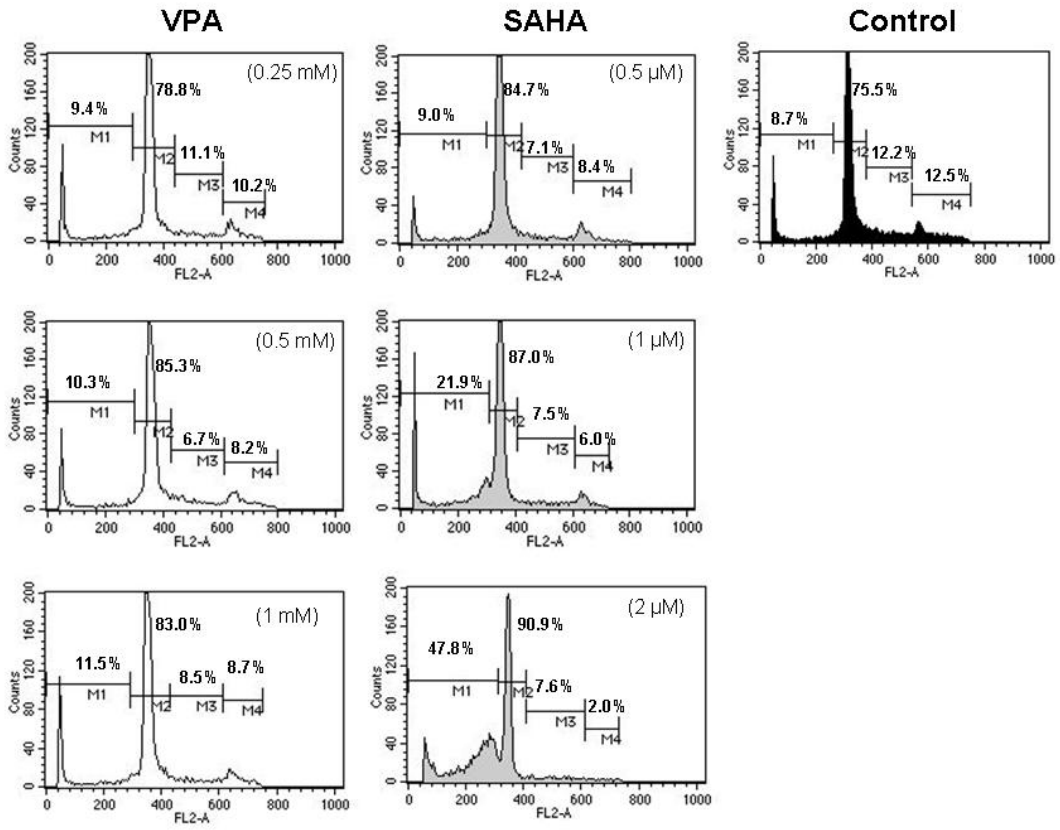


Figure 3.6: Viabilty and proliferation of NK cells after exposure to HDACi. Primary NK cells from healthy donors were treated simultaneously with 100 U/ml IL-2 and either VPA or SAHA at indicated concentrations for 4 days. The effect of HDACi on cell cycle was determined by staining cells with PI. M1, M2, M3 and M4 indicate sub G1-, G0/G1-, S- and G2/M-phases respectively; empty histograms represent VPA treated cells; grey histograms represent SAHA treated cells; black histogram represents untreated (control) NK cells; numbers in parentheses indicate respective concentrations of HDACi used; values represent percentage of cells in the different phases. The percentages of cells in G0/G1, S and G2/M phases were deduced from the number of viable cells (set to 100%) after subtracting the dead cells (sub G1) from total gated cells. One representative of 3 different experiments is shown.

3.6 HDACi suppress IL-2-mediated NK cell cytotoxicity

The role of HDACi on NK cell cytotoxicity was next investigated. IL-2-activated NK cells were treated with either SAHA or VPA for 4 days after which the cytotoxicity against K562 cells was determined using a 4 h CLM assay. Interestingly, both SAHA and VPA dramatically suppressed IL-2-activated NK cell cytotoxicity in an E:T cell ratio-dependent

manner (Fig. 3.7a). Decreased NK lytic activity of HDACi treated NK cells was also observed in other leukemic cell lines including Jurkat T cells and HL-60 cells (Fig. 3.7b). A 24 h pretreatment of Jurkat T cells and HL-60 cells with SAHA resulted in almost 60% (54% vs. 86%) and 14% (57% vs. 65%) increased NK cell-mediated lysis in SAHA treated Jurkat and HL-60 cells respectively. The increased NK cell lysis was however suppressed when SAHA treated NK cells were used as effector cells (Fig. 3.7b). Taken together, Jurkat T cells and HL-60 cells become more susceptible to NK cell-mediated lysis upon exposure to SAHA while NK cell activity gets repressed upon treatment with SAHA. In contrast, treatment of K562 for 24 h with 1 µM SAHA decreased its susceptibility to NK cell lysis by 50% [Fig. 3.7b].

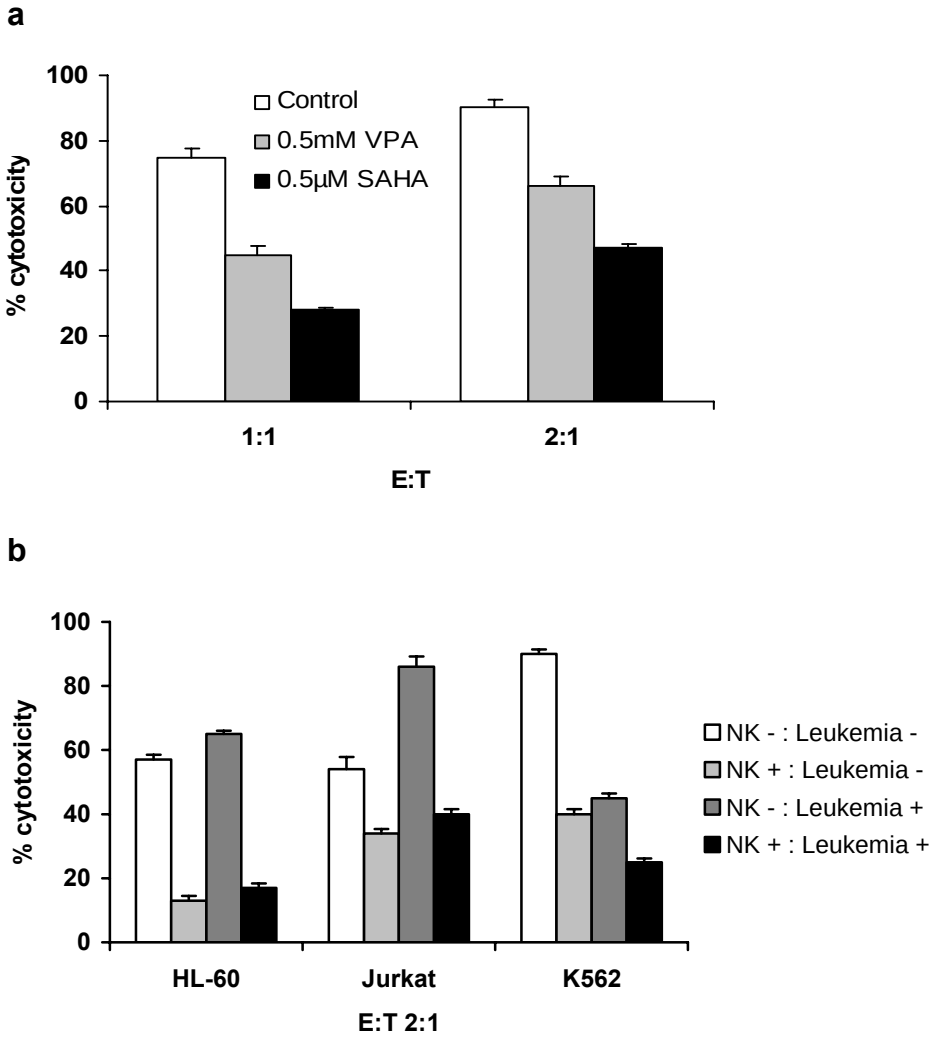


Figure 3.7: HDACi suppress NK cell cytotoxicity. Primary NK cells from healthy donors were treated simultaneously with 100 U/ml IL-2 and either 0.5 mM VPA or 0.5 μM SAHA for 4 days. Primary NK cells treated only with 100 U/ml IL-2 were used as control. [a] A 4 h NK cell cytotoxicity assay against K562 target cells was performed at indicated E:T ratios. Columns represent means of triplicate of one representative experiment; error bars indicate ± SD. [b] A 4 h NK cell cytotoxicity assay against K562, Jurkat T cells and HL-60 cells, pretreated with 1 μM SAHA for 24 h at an E:T ratio of 2:1 was carried out. [-] indicate without SAHA [+] indicate with SAHA. Columns represent means of triplicate of one representative experiment; error bars indicate ± SD.

3.7 HDACi down-modulate NK cell activating receptors expression

NK cell cytotoxicity is a complex process that requires adhesion to target cells, synapse formation and signal transduction leading to granule polarization and exocytosis. Accordingly, it is conceivable that HDACi might interfere with different steps in the process. To address these issues, the surface expression patterns of NKp30, NKp44, NKp46, NKG2D and DNAM-1, NKG2A and KIR in untreated as well as in SAHA and VPA treated NK cells were investigated. A correlation between NK cell cytotoxicity and NK cell receptor expression pattern was observed. The lytic capacity of NK cells treated with HDACi was associated with a high decreased surface expression of NKp30 and NKp46 while NKp44, NKG2D and DNAM-1 were not significantly changed. No changes were observed in the surface expression of KIR and NKG2A inhibitory receptors (Fig. 3.8a). To show whether the expressions of NKp46 and NKp30 are also influenced at the transcriptional level upon HDACi treatment, we examined the gene expression patterns of NKp46 and NKp30 in untreated and HDACi treated NK cells. Real-time RT-PCR results revealed a 5- and 9-fold decrease as well as a 9- and 12-fold decrease expression in NKp46 and NKp30 respectively for VPA and SAHA treated NK cells when compared to untreated NK cells (Fig. 3.8b).

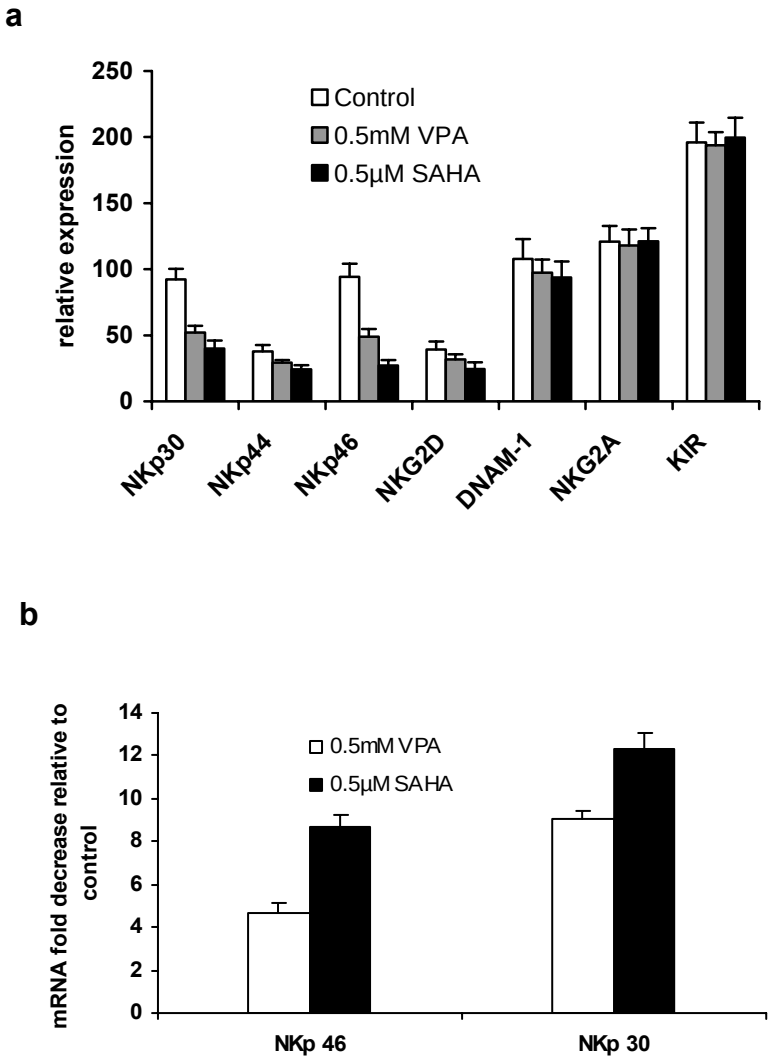


Figure 3.8: HDACi down-modulate expression of NK cell activating receptors. Primary NK cells from healthy donors were treated simultaneously with 100 U/ml IL-2 and either 0.5 mM VPA or 0.5 µM SAHA for 4 days. Primary NK cells treated only with 100 U/ml IL-2 were used as control. [a] Flow cytometric analysis for the expression of indicated NK cell activating or inhibitory receptors. Columns indicate relative expression of one representative of at least five separate experiments. [b] Real-time RT-PCR for the mRNA expression levels of the different transcripts. Data are expressed as fold decrease of mRNA expression in VPA or SAHA treated NK cells relative to untreated (control) NK cells. Histograms are representative of results obtained with NK cells derived from 3 different donors. Each experiment was run in duplicate; error bars indicate ± SD.

HDACi suppressed NK cell activity and NCR surface expression only when added simultaneously with IL-2, while they did not influence NK cells cultured without IL-2 (Fig.

3.9). Basically NK cell activating/inhibitory receptor expression levels were 3- to 4-fold higher in IL-2 cultured NK cells than in NK cells cultured without IL-2.

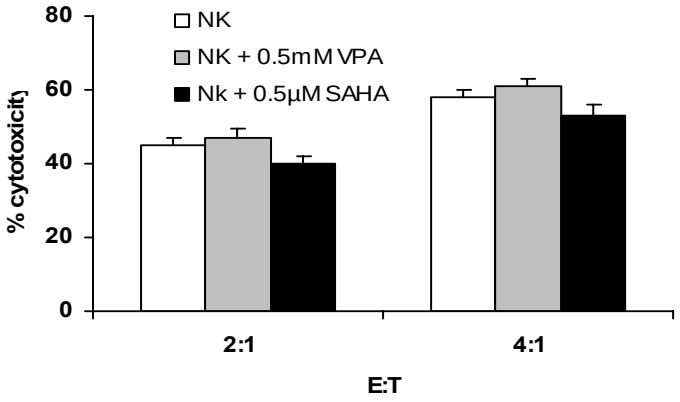


Figure 3.9: HDACi does not suppress cytotoxicity of NK cells not treated with IL-2. Primary NK cells from healthy donors were treated with either 0.5 mM VPA or 0.5 µM SAHA for 4 days. Primary NK cells treated without IL-2 were used as control. A 4 h NK cell cytotoxicity assay against K562 target cells was performed at indicated E:T ratios. Columns represent means of triplicate of one representative experiment; error bars indicate ± SD.

Since NK cell cytolysis also depends on binding mediated by adhesion molecules like LFA-1, effects of HDACi on LFA-1 surface expression were determined. It was also determined whether HDACi influence IL-2 receptor since only NK cells cultured with IL-2 showed impaired activity upon HDACi treatment. HDACi did not significantly modify the expression neither of LFA-1 nor of IL-2 receptors (CD25, CD122 and CD132) on NK cells (Table 3.1).

Table 3.1: Flow cytometric analysis for the expression of indicated receptors

	relative expression[a]		
Receptors	NK+IL-2	NK + IL-2 + 0.5 mM VPA	NK + IL-2 + 0.5 μM SAHA
LFA-1	20 ± 2	23 ± 3	19 ± 2.5
CD25	37 ± 3.3	34 ± 4	40 ± 1
CD122	15 ± 4	17 ± 2	14 ± 3
CD132	26 ± 2.5	22 ± 4.5	28 ± 2

[a] Results are expressed as relative fluorescent unit ± SD of triplicates of one representative experiment.

These results suggest that HDACi act directly on selected NK cell receptors rather than by interfering with the ability of NK cells to respond to IL-2 or NK cell binding to target cells.

3.8 HDACi suppress NK cell function

To assess whether HDACi-induced modulation of NKp46 and NKp30 receptors resulted in an alteration of NK cell activity, treated and untreated NK cells were compared in a redirected killing assay against FcγR$^+$ P815 target cell line. The FcγR$^+$ P815 cell line has been extensively used for mAb-mediated redirected killing assays using NK cells and mAbs capable of triggering their cytolytic functions [220-222]. This would allow the assessment in a cytolytic assay the direct effect of VPA and SAHA on the specific activity of the trigerring receptors NKp46 and NKp30. As shown in Fig. 3.10, treatment of NK cells with VPA and SAHA clearly reduced the ability of anti-NKp46 and anti-NKp30 mAbs to induce NK cell-mediated lysis.

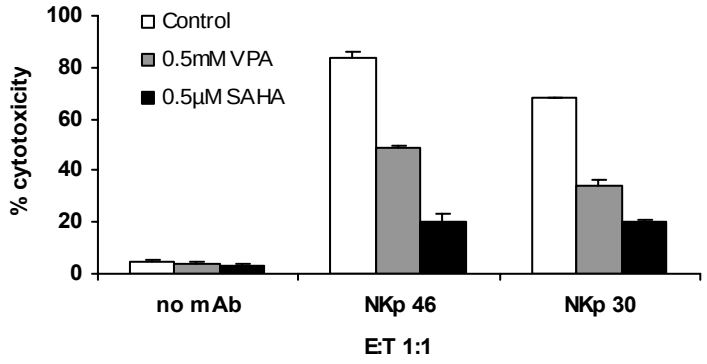


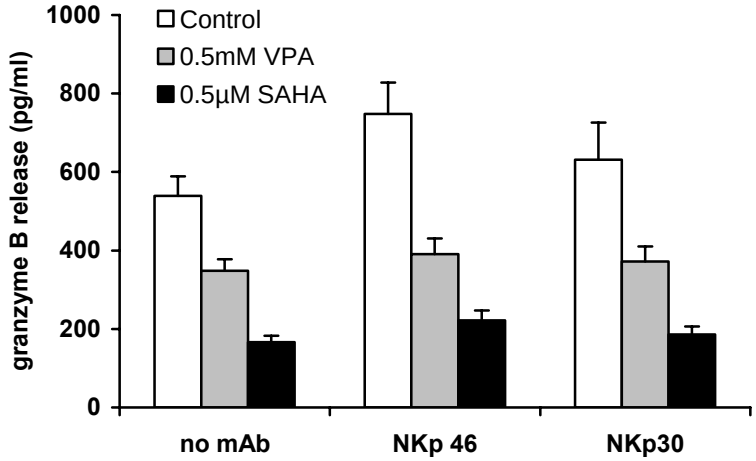
Figure 3.10: HDACi suppress NK cell function. A 4 h NK cell cytotoxicity was assessed in a redirected killing assay against the FcγR$^+$ P815 target cell line either in the presence or absence of mAbs to the indicated receptors. Columns represent means of triplicate of one representative experiment; error bars indicate ± SD.

These findings suggest that HDACi may affect NK cell function by interfering with the expression and the function of NKp46 and NKp30 triggering receptors.

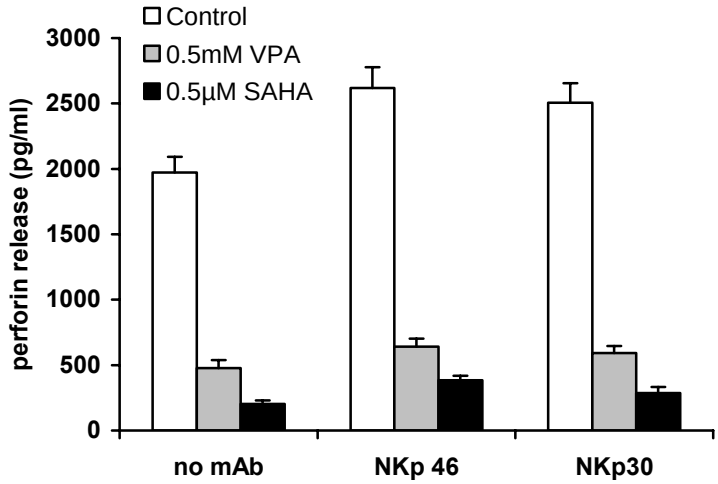
3.9 HDACi impair granule exocytosis and inhibit IFN-γ production

To validate the effect of HDACi on NKp46 and NKp30 expression and function, perforin and granzyme B degranulation after cross-linking of NKp46 and NKp30 with mAbs was analyzed. As shown in Fig. 3.11a, an impaired granzyme B release was observed upon treatment of NK cells with VPA and SAHA. Cross-linking of NK cells with mAbs resulted in increased granzyme B release, further indicating the direct effect of HDACi on NKp46 and NKp30. Similar results were obtained for perforin degranulation (Fig. 3.11b). It is worthy to mention that intracellular perforin and granzyme B expressions using flow cytometry were not significantly affected by HDACi treatment (Fig. 3.11c). Furthermore, treatment of IL-2-activated NK cells for 72 h with either VPA or SAHA also inhibited IFN-γ production (Fig. 3.11d).

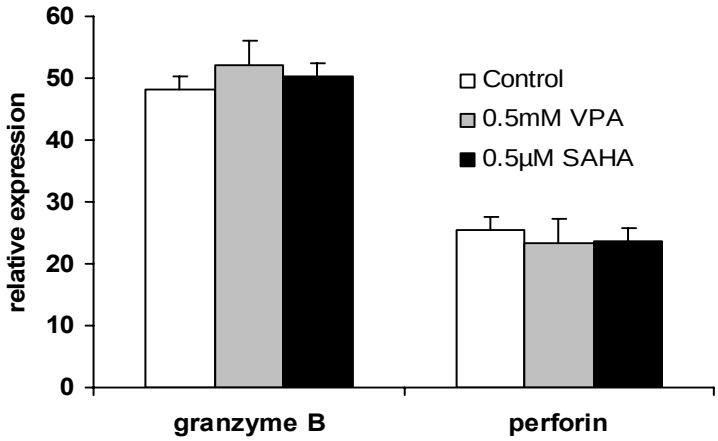
a


granzyme B release (pg/ml)
1000
800
600
400
200
0
Control
0.5mM VPA
0.5µM SAHA
no mAb
NKp 46
NKp30

b


perforin release (pg/ml)
3000
2500
2000
1500
1000
500
0
Control
0.5mM VPA
0.5µM SAHA
no mAb
NKp 46
NKp30

c


relative expression
60
50
40
30
20
10
0
Control
0.5mM VPA
0.5µM SAHA
granzyme B
perforin

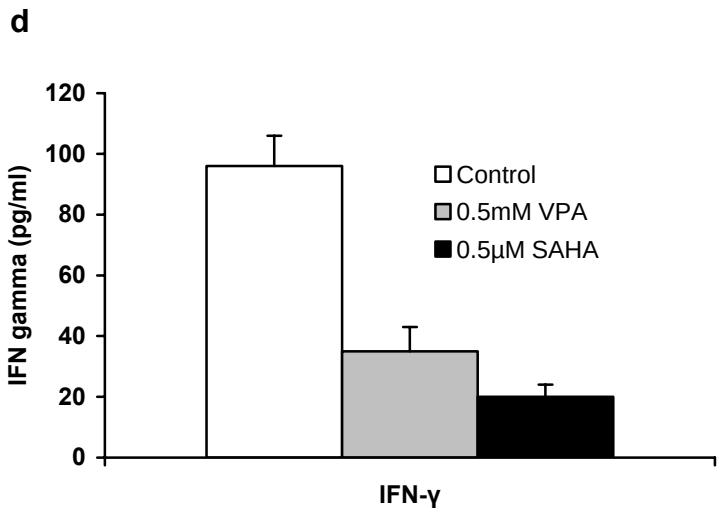


Figure 3.11: HDACi impair granule exocytosis and inhibit IFN-γ production. Cells were stimulated by cross-linking the indicated NK receptors with appropriate mAbs. After overnight incubation at 37°C in IMDM + 10% FBS alone, supernatants were collected and analyzed in an ELISA assay specific for *in vitro* quantitative determination of [a] granzyme B and [b] perforin release. Columns indicate granzyme B or perforin granule release (pg/ml). [c] Flow cytometric analysis for intracellular granzyme B and perforin expression. [d] IL-2-activated NK cells were treated for 72 h with or without VPA and SAHA, after which IFN-γ production was measured by ELISA. One representative of at least three separate experiments is shown; error bars indicate ± SD.

3.10 SAHA and VPA suppress NFκB activation in IL-2-activated NK cells

It was previously reported by Zhou et al [223] and Kim et al [224] that IL-2 increases NK cell cytotoxicity and proliferation through activation of NFκB signaling pathway. To verify a possible role of NFκB in this experiment, the effect of HDACi on NFκB binding was compared with that of a potent NFκB inhibitor BAY 11-7085. NK cells were cultured simultaneously for 4 days with 100 U/ml IL-2 and either HDACi or BAY 11-7085. NFκB activation was then measured. BAY 11-7085 as well as SAHA and VPA inhibited NFκB activity (Fig. 3.12a). BAY 11-7085 (not toxic to NK cells at concentration used- trypan blue exclusion counts) also abrogated NK cell lysis of K562 and suppressed surface expression of NK cell activating receptors NKp30, NKp44, NKp46 and inhibitory receptor NKG2A (Fig.

3.12b). These results suggest that HDACi prevent IL-2-activated NK cell cytotoxicity by suppressing NK cell activating receptors in association with the inhibition of NFκB activation.

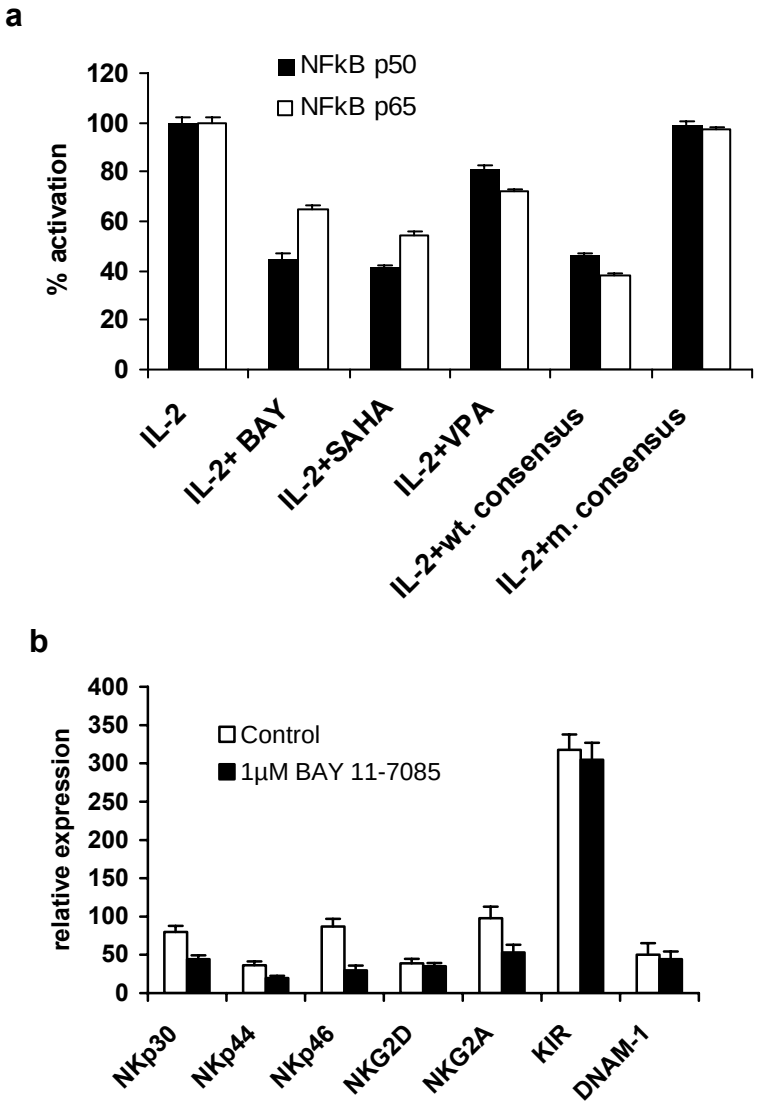


Figure 3.12: HDACi prevent NFκB activation. Primary NK cells from healthy donors were treated simultaneously with 100 U/ml IL-2 and either 0.5 mM VPA or 0.5 μM SAHA or 1 μM BAY 11-7085 for 4 days. Primary NK cells treated only with 100 U/ml IL-2 were used as control. [a] NFκB activation assay was performed as described in materials and methods. The relative luminescent values of IL-2 alone were set to 100%, from which the percentage activation of other treated NK cells were deduced. [m] indicate mutated consensus oligonucleotide, [wt] indicate wild-type consensus oligonucleotide. Columns represent means of triplicate of one representative experiment; error bars indicate ± SD. [b] Flow cytometric analysis for the

expression of indicated NK cell activating or inhibitory receptors upon treatment with BAY 11-7085. Columns indicate relative expression of one representative of at least five separate experiments; error bars indicate ± SD.

3.11 Viability of leukemic cells upon araC treatment

MTT assay was performed to determine the viability of the leukemic cell lines used upon treatment with araC. Cytotoxic effects of araC, expressed as concetrations inhibiting 50% of cell growth (IC_{50}) were decreased in araC-resistant cells when compared with parental cells. Resistance index (RI, ratio of IC_{50} in $H9^r100^{ARAC}$ and H9 cells as well as in Molt-4^r100^{ARAC} and Molt-4 cells) of araC for H9 and Molt-4 cells were respectively 2.2×10^4 and 4.3×10^4 (Table 3.2).

Table 3.2: Cytotoxic effects of araC in Molt-4, Molt-4^r100^{ARAC}, H9 and $H9^r100^{ARAC}$ cells

Cell line	IC_{50} (µM)[a]		
	Parental	Resistant	RI[b]
Molt-4	0.029 ± 0.0018	1256 ± 234	4.3×10^4
H9	0.034 ± 0.0047	756.8 ± 53.7	2.2×10^4

[a] Results represent mean values ± SD of three different experiments. [b] RI (Ratio IC_{50} resistant, IC_{50} parental cell lines).

3.12 Cytotoxic activity of IL-2-activated NK cells against leukemic cell lines

The cytolytic activity of IL-2-activated NK cells against parental and araC-resistant H9 and Molt-4 cell lines was determined. The results show that NK cells effectively kill parental H9 and Molt-4 cell lines. Both araC-resistant cell lines showed higher sensitivity to NK cell lysis than parental cell lines (at E:T 4:1, 55% vs. 38% respectively for araC-resistant H9 and H9 cells; 70% vs. 31% respectively for araC-resistant Molt-4 and Molt-4 cells; Fig. 3.13). The increased sensitivity of araC-resistant leukemic cells than parental cells was neither due to the

direct cytotoxic effect of araC on the cells nor the direct effects of araC on cell metabolism since araC-resistant cells used for the experiment were cultured for up to 10 subcultures without the drug. NK cell lysis of K562 cell line was used as positive control and it resulted in about 90% lysis at E:T 4:1.

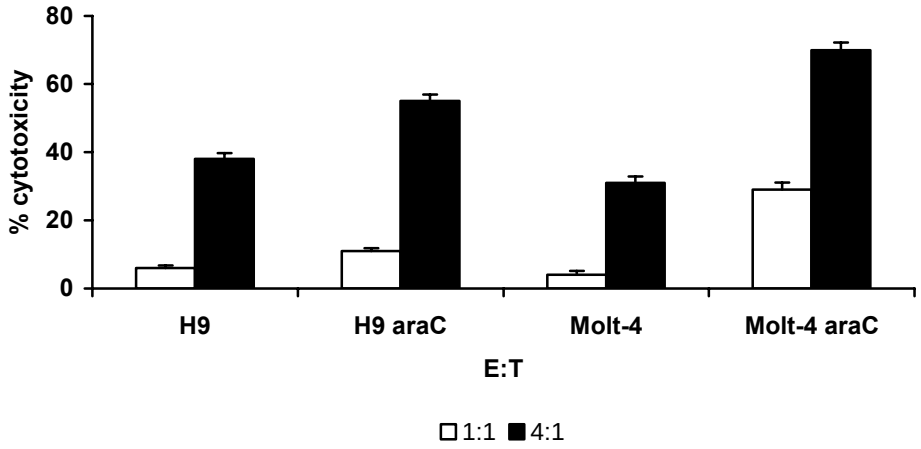


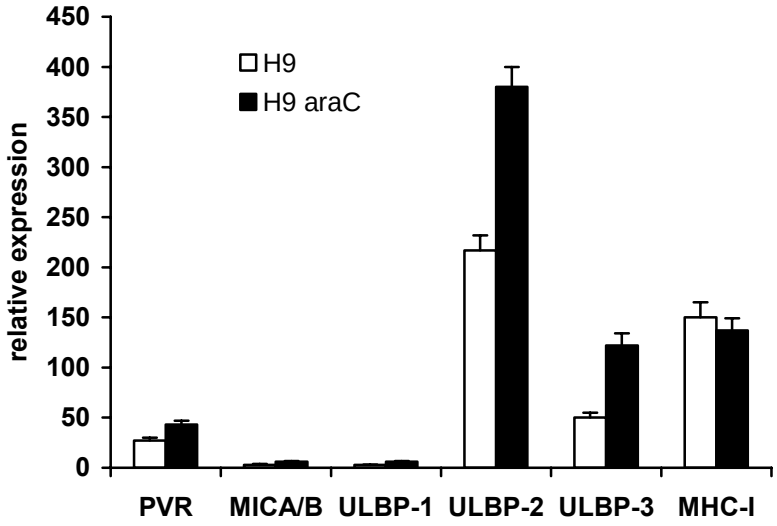
Figure 3.13: NK cell cytotoxicity of parental and araC-resistant leukemic cells. Killing of H9 and Molt-4 cell lines and their araC-resistant counterparts was assessed by G3PDH release. Five thousand target cells were coincubated with IL-2-activated NK cells at the indicated E:T ratios for 4 h at 37°C. The results are mean ± SD of 3 independent experiments.

3.13 Expression of NK cell activating and inhibitory ligands in leukemic cells

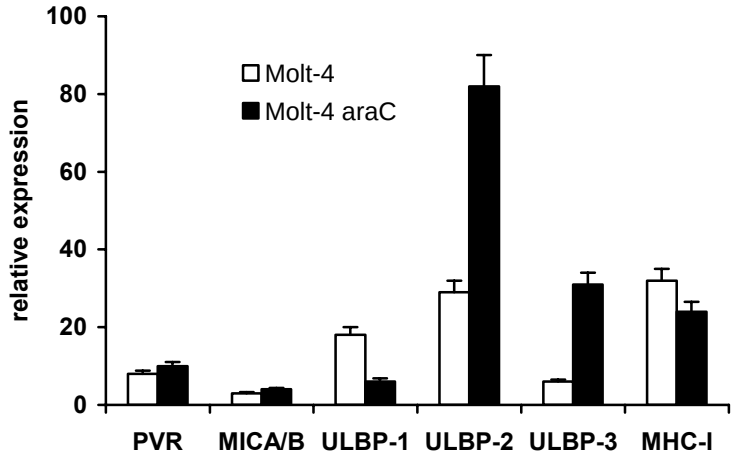
The expression pattern of ligands for the NK cell activating/inhibitory receptor in both parental and araC-resistant cell lines was investigated to show whether it correlates with the lysis of leukemic cells by NK cells. The observed increased lysis was associated with a corresponding increase in the cell surface expression of ULBP-2 and ULBP-3 in araC-resistant H9 cells (relative expression: 380 vs. 217 for ULBP-2, 122 vs. 50 for ULBP-3) and Molt-4 cells (82 vs. 29 for ULBP-2, 31 vs. 6 for ULBP-3; Fig. 3.14a and b). There was no significant change in MHC-I expression level in both parental and araC-resistant H9 and Molt-4 cell lines. The mRNA expression of araC-resistant and parental cell lines used for this study was also evaluated using real time RT-PCR. As shown in Fig. 3.14c, an increased

ULBP-2 and ULBP-3 mRNA expression was observed in araC-resistant cell lines (1.57-fold ULBP-2, 5.93-fold ULBP-3 increase for araC-resistant H9 and 5.98-fold ULBP-2, 2.13-fold ULBP-3 for araC-resistant Molt-4) in comparison to their respective parental cell lines. This indicates that araC modifies NK cell activating receptor ligand expression at the transcriptional level. The effect of this modification appears to be maintained since several passaging of leukemic cell (at least 10 passages without araC treatment) continued to display this effect.

a



b



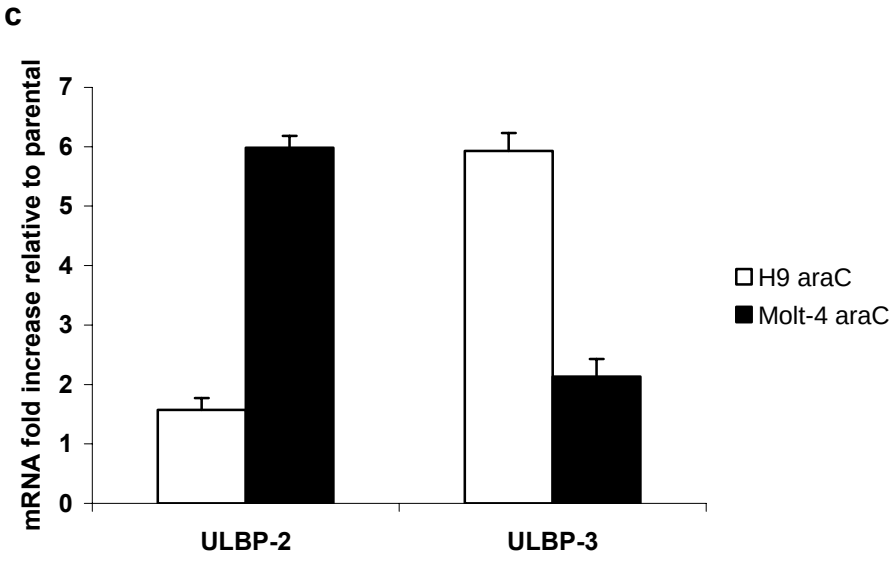


Figure 3.14: Expression of ligands for NK cell receptors in leukemic cells. [a] Flow cytometric analysis for the expression of ligands in [a] parental and araC-resistant H9 cells, and [b] parental and araC-resistant Molt-4 cells. Columns indicate relative expression of one representative of at least 3 separate experiments; error bars indicate ± SD. [c] Real-time RT-PCR for the mRNA expression levels of ULBP-2 and ULBP-3 transcripts. Data are expressed as mRNA fold increase in araC-resistant H9 and Molt-4 cells relative to parental H9 and Molt-4 cells. Histograms are representative of three different experiments. Each experiment was run in duplicate; error bars indicate ± SD.

3.14 NK cell recognition of leukemic cell lines via NKG2D

In an attempt to understand a possible mechanism of the increased NK cell lysis of resistant leukemic cells as well as the functionality of NKG2D ligands, mAb masking experiments were performed. For this purpose, parental as well as araC-resistant H9 cell lines were selected for a 4 h cytotoxicity assay, since they displayed increased sensitivity to NK cell-mediated cytotoxicity as well as increased NKG2D ligand densities on their cell surfaces (80% increase in ULBP-2 surface expression in all araC-resistant cell lines used). As shown in Fig. 3.15, blocking PVR on parental and araC-resistant H9 cells did not show significant decrease in NK cell lysis (36% vs. 33%, 58% vs. 50% respectively for H9 and araC-resistant H9 cells). However blocking ULBP-2 or ULBP-3 alone showed a strong inhibition of NK cell

lysis (36% vs. 3% ULBP-2 and 4% ULBP-3, 58% vs. 4% ULBP-2 and 15% ULBP-3 respectively for H9 and araC-resistant H9 cells). It is worthy of note that in parental H9 cell line, the expression level of ULBP-2 and ULBP-3 was 8-fold and 1.9-fold respectively higher than that of PVR, while in araC-resistant H9 cell line the expression level of ULBP-2 and ULBP-3 was approximately 8.8 and 2.8-fold respectively higher than that of PVR. Combined blocking of ULBP-2 and ULBP-3 on araC-resistant H9 cells resulted in total abrogation of NK cell lysis. A 100% inhibition of NK cell lysis was also observed upon blocking NKG2D on NK cells (Fig. 3.15). The results suggest that NK cell activation via NKG2D receptor-ligand binding is the possible mechanism involved in the increased lysis of araC-resistant H9 cell line.

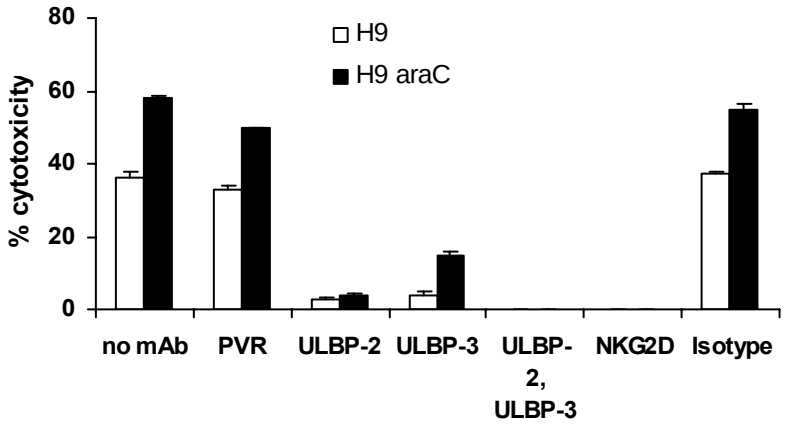


Figure 3.15: NK cells recognize leukemic cells via NKG2D receptor. Five thousand H9 and araC-resistant H9 cells were coincubated with IL-2-activated NK cells at E:T ratio 4:1 for 4 h at 37°C either in the absence or presence of 10 µg/ml mAb. IgG isotype control was used as negative control. The results are mean ± SD of 3 independent experiments.

3.15 Possible mechanism of increased ligand expression in araC-resistant leukemic cells

ERK and AKT may influence sensitivity of leukemic cells to araC [225-227]. Moreover, both ERK and AKT were shown to be involved in the regulation of expression of NKG2D ligands [199, 228]. For these reasons, both ERK and AKT signaling pathways were studied for their

constitutive activation status in both parental and araC-resistant cells. Western blot analyses using parental H9 as well as araC-resistant H9 cells showed a stronger constitutive phosphorylated ERK but not AKT in araC-resistant H9 cells. Constitutive activation of ERK1 (p44) was particularly diminished in parental H9 cells (Fig. 3.16a). The enhanced constitutive phosphorylated ERK in araC-resistant H9 cells was confirmed by FACS analysis (relative expression: 229 ± 12 vs. 186 ± 14 for araC-resistant H9 and parental H9 cells respectively; Fig 3.16b).

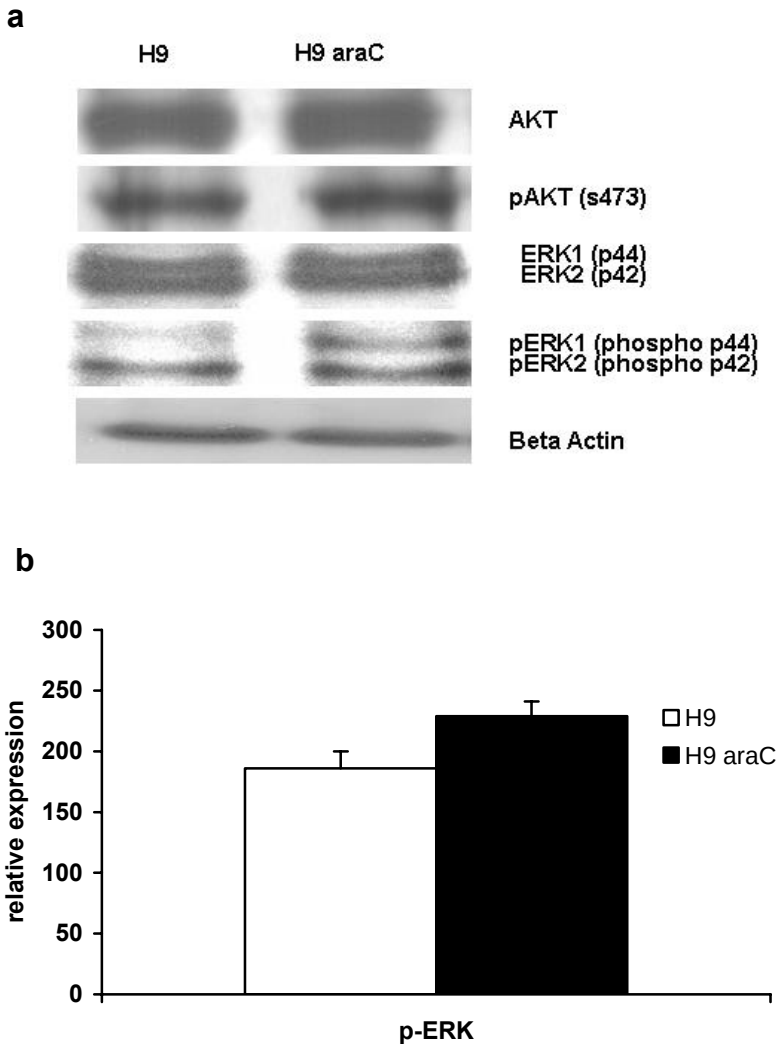
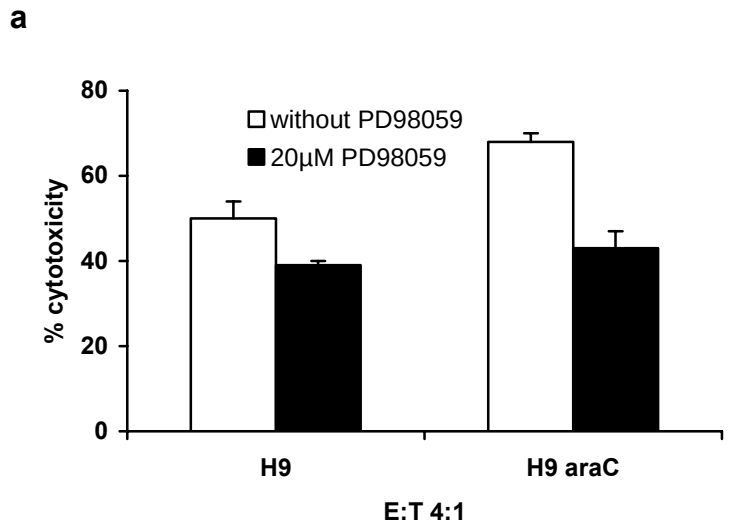


Figure 3.16: Mechanism of increased ligand expression in araC-resistant cells. [a] The same amount of cell extracts prepared from the same passage of H9 and araC-resistant H9 cell cultures was used for western blot analyses. Constitutive AKT and ERK activation was assessed using antibodies that recognize AKT phosphorylated at ser-473 and ERK1/2 phosphorylated at Thr202/Tyr204. [b] Flow cytometric analysis for

constitutive phosphorylated ERK expression. Data are representative of at least three experiments, error bars indicate ± SD.

3.16 Role of ERK signaling in NKG2D ligand expression

To verify the role of ERK signaling pathway in NKG2D ligand expression, FACS analysis and cytotoxicity assay were performed upon ERK inhibition of parental and araC-resistant H9 cells using 20 μM of the ERK activation inhibitor PD98059. Treatment of parental H9 and araC-resistant H9 cells with PD98059 impaired NK cell lysis (from 50% to 39% and 68% to 43% for H9 and araC-resistant H9 respectively; Fig. 3.17a) and decreased ULBP-2 (relative expression from 182 to 152 and 266 to 102 for H9 and araC-resistant H9 respectively) and ULBP-3 (relative expression from 24 to 14 and 60 to 49 for H9 and araC-resistant H9 respectively) expression (Fig. 3.17b). Taken together, these results demonstrate that increased sensitivity of araC-resistant leukemic cells to NK cell lysis is due to higher NKG2D ligand expression, resulting from more active ERK but not AKT signaling pathway.



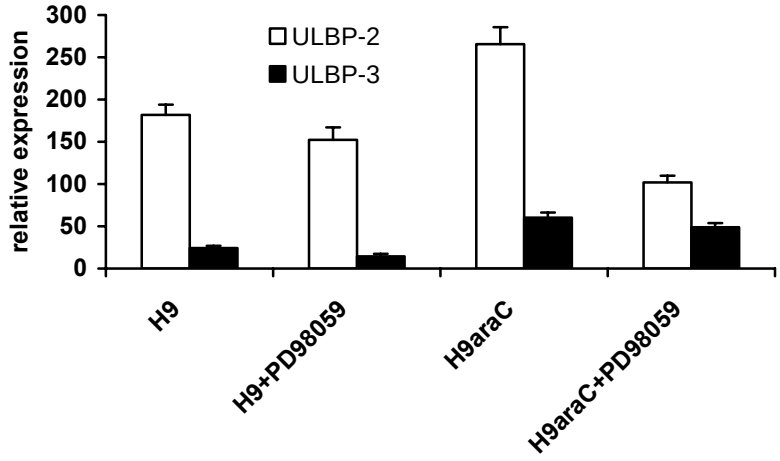


Figure 3.17: ERK signaling in NKG2D ligand expression. Parental and resistant cells were treated for 48 h with the ERK activation inhibitor PD98059. [a] IL-2-activated NK cell cytotoxicty and [b] flow cytometric analysis for ULBP-2 and ULBP-3 expression were determined. Results are representative of 3 different experimens. Error bars indicate ± SD.

4.0 Discussion

4.1 Luminescent assay

Properly designed coupled luminescent assays are able to combine the advantages of specific assays for enzyme function with the very great sensitivity of luminescent detection methods. In coupled luminescent assays, the inherent sensitivity of luciferase detection is enhanced by the amplification effect of enzyme turnover, which produces thousands, millions, or billions of high-energy molecules for each molecule of enzyme. The luminescent signal is produced by firefly luciferase acting on adenosine triphosphate (ATP), which in turn is produced by the coupled reactions of G3PDH and phosphoglycerokinase (PGK), two consecutive enzymes of the glycolytic pathway. G3PDH, a very abundant enzyme in all known cells, is measured to quantify release (and therefore cell death and/or membrane damage). The fact that G3PDH is a natural component of cells, and does not need to be introduced into the cells in any manner, distinguishes this assay from all methods which require prelabeling of the cells, or transfection, transformation, or other methods of introducing proteins or other molecules into the target cells in order to generate a signal in a later step [196].

The G3PDH /PGK/luciferase (GPL) coupled luminescent assay introduced by Corey et al [196] addressed several problems of the methods mentioned above. Nevertheless, the GPL assay is cumbersome to execute in that it involves four transfer steps (cocktail to reaction vessel, sample to reaction vessel, luciferase to luminance vessel, aliquot of reaction to luciferase) and two incubations prior to actual read. Moreover, since the assay is not compatible with live cells, live cells need to be separated from the supernatant by centrifugation prior to the assay.

4.2 Establishing CLM to measure NK cell cytotoxicity

CLM enjoys the features of GPL assay. In contrast to the GPL assay, CLM is a one-step homogeneous system in which the sample being tested is mixed with the reagent cocktail and neither separation of live cells from supernatant nor further transfers are required prior to readout.

CLM was established by measuring the cytolytic activity of IL-2-activated NK cells against K562. To validate CLM, a NB model was used. Most NB cells are generally resistant to NK cell lysis [144] when compared to K562 that are readily lysed even with NK cells that have not been activated with lymphokine such as IL-2. All NB cell lines tested were resistant to non-activated NK lysis, but were readily lysed by IL-2-activated NK cells. This may be due at least in part to the failure of NB cells to express NKG2D ligands, as were observed for MICA/B in the present study. This is consistent with previous report which showed that NKG2D-activating receptor ligands on the surface of primary neuroblasts and NB cell lines are down-regulated [229, 230]. On the other hand, PVR was readily expressed by NB cells. PVR, a ligand recognized by DNAM-1 receptor [215, 217], was used in mAb blocking experiments to further validate CLM. DNAM-1 (CD226) which is expressed in virtually all human NK cells, T cells, monocytes, platelets and a subset of B-lymphocytes, is another surface molecule that has been shown to participate in the induction phase of NK cell activation. DNAM-1 is known to be involved not only in NK cell activation but also in cell-cell adhesion [231-233]. This suggests that the adhesion of NK cells to NB cells could be mediated by DNAM-1-PVR interaction. The present results support previous observations [215] demonstrating that the assessment of PVR expression could be used as an immunological marker for the susceptibility of NB cells to NK cell-mediated attack.

Interestingly, the higher susceptibility of drug resistant NB cells to NK lysis than non-resistant cells is shown in this report with CLM. UKF-NB-4, which possesses the intrinsic multidrug resistant (MDR) phenotype [234], and the drug-induced resistant UKF-NB-$2^{r}VCR^{10}$ [202] cell line were more susceptible to NK cell-mediated lysis than the parental UKF-NB-2 and UKF-NB-3 cell lines. Also, araC-resistant leukemic cell lines were also more sensitive to NK cell lysis than their parental cell lines. This is of interest since induction of drug resistance in tumor cells as shown seems to be associated with changes in their sensitivity to NK cell-mediated lysis. Some studies demonstrated that multidrug resistant leukemic cells develop decreased sensitivity to NK cell-mediated lysis mainly at the level of killer/target recognition [235, 236]. On the other hand, multidrug resistant cells derived from some solid tumors may exert increased sensitivity to NK cell-mediated lysis by different mechanisms [237, 238]. The increased sensitivity of multidrug resistant NB cells and leukemic cells might suggest a role for drug resistance in NK cell-mediated cytotoxicity.

CLM is a highly sensitive, safe and fast method to determine NK cell cytotoxicity. The features of CLM may be of particular importance in cases with small blood samples including pediatric patients with solid tumors or viral infections.

4.3 SAHA and VPA suppress IL-2-mediated NK cell cytotoxicity

Several reports have described the direct inhibition of NK cell function by several substances including cortisol, methylprednisolone, adiponectin, $_{L}$-kynurenine, and All-*trans* retinoic acid (ATRA) [214, 220, 221, 224, 239, 240]. The glucocorticoid cortisol at concentrations up to 2 µM was shown to repress the synthesis of both perforin mRNA and granzyme A in NK3.3 cell line [239]. Cortisol also down-regulated LFA-1 and inhibited conjugate formation of NK3.3 cells with their target K562 cells, thereby completely abrogating cytotoxic function of NK3.3 cell line [239]. The corticosteroid methylprednisolone impaired the expression of

NKp30, NKp44 and NKp46 both in NK cells from patients undergoing allogenic bone marrow transplantation and in IL-2-activated NK cells from healthy individuals [220]. In the report by Chiossone et al [214] methylprednisolone was shown to inhibit NKp30 and NKp44 surface expression in IL-2 and IL-15-cultured NK cells. Tyr phosphorylation of STAT1, STAT3 and STAT5 was also inhibited. Impairment of NK cell cytotoxicity was observed to strictly correlate with the inhibition of ERK1/2 phosphorylation and perforin release in IL-2 and IL-15-cultured NK cells [214]. On the other hand, adiponectin suppressed IL-2-induced NK cell cytotoxicity by inhibiting NFκB signaling pathway and down-regulating IFN-γ, Fas Ligand (FasL) and TRAIL expression in NK cells [224]. The inhibition of surface expression of NKp46 and NKG2D as well as impairment of NK cell ability to kill target cells recognized via NKp46 and NKG2D was reported for the tryptophan catabolite $_{L}$-kynurenine [221]. The cytotoxic activity of the NK cell line NK92 was also shown to be inhibited by ATRA. The researchers reported that ATRA suppressed NFκB activity and prevented the degradation of IκB, inhibited IFN-γ production and gene expression of granzyme B, NKp46 and NKp30 [240]. Reports of the direct effect of HDACi on NK cell cytotoxicity however do not exist.

The results shown in this study provide for the first time evidence that treatment of NK cells with HDACi can suppress their lytic activity against leukemic cells. NK cell inhibitory effects were associated with the suppression of surface expression and function of specific triggering receptors (NKp46 and NKp30) responsible for the induction of NK cell-mediated cytotoxicity. This inhibitory effect was also effective at the transcriptional level. Moreover, inhibition of IFN-γ production and impaired granule release were observed upon treatment of NK cells with VPA and SAHA. After cross-linking of NK cells with NKp46 and NKp30 granzyme B and perforin granule release were increased, further indicating the direct effect of HDACi on NKp46 and NKp30. HDACi acted directly on selected NK cell receptors rather than by interfering with the ability of NK cells to respond to IL-2 since IL-2R expression

were not affected. On the other hand, surface expression levels of inhibitory receptors including KIR and NKG2A were not influenced by HDACi treatment, indicating a specific effect of HDACi on NK cell triggering receptors.

4.4 HDACi-treated NK cells repress HDACi-induced enhanced NK cell sensitivity of leukemic cells

Independent reports by Cinatl et al [144], Skov et al [146], and Armeanu et al [147] described an increased NK cell-mediated lysis of certain tumors upon treatment with VPA and SAHA. Although the present study found in concert with these reports [144, 146, 147] that treatment of leukemic cells with HDACi increases their sensitivity to NK cell lysis, the increased sensitivity was however reversed when NK cells were pretreated with SAHA. These findings suggest that direct inhibitory effect of HDACi on lytic NK cells may overweigh the HDACi-induced increased sensitivity of leukemic cells to NK cell lysis.

4.5 Mechanism of HDACi inhibition of NK cell cytolytic activity

Optimal NK cell development and activation as well as cytolytic activity involve IL-2Rβ signals that also up-regulate expression of the pore-forming effector molecules perforin/granzyme [241-243]. The activation of NFκB by IL-2R signaling and its role in perforin regulation in NK cells was investigated by Zhou et al [223]. They demonstrated that IL-2-induced up-regulation of perforin in primary NK cells and in a model cell line (NK3.3) was blocked by two pharmacological inhibitors of NFκB activation. Direct evidence for the activation of the NFκB pathway by IL-2R signals in NK cells was shown to involve activation of IKKα kinase, IκB degradation, nuclear translocation of p50/65 complexes, and ultimately, transcriptional activation of the perforin gene via an NFκB binding element in its upstream enhancer [223]. Furthermore, NFκB proteins were shown to regulate the expression of genes involved in immune and inflammatory responses including IFN-γ and granzyme B [244, 245].

Blanchard and Chipoy [97] reviewed several reports demonstrating the inhibition of NFκB transcriptional activity after treatment with HDACi. Here, mechanisms of NFκB transcriptional inhibition by HDACi including inhibition of nuclear translocation and DNA binding of NFκB were illustrated [97]. Since HDACi did not modify IL-2R in this research, it is conceivable that HDACi might not interfere with IL-2 signaling in NK cells but rather directly modulate NFκB activity. In fact, some studies revealed that prevention of NFκB activity by pharmacological treatments [224] or a defective NFκB activation in patients with the genetic disorder hypohidrotic ectodermal dysplasia [246] leads to a deficient NK cell cytotoxicity. In concordance, the present findings strongly indicate that inhibition of NFκB activation by SAHA and VPA is an important molecular mechanism by which HDACi suppress NK cell cytotoxicity.

4.6 AraC-induced resistance of leukemic cells increases their sensitivity to NK cell lysis

Numerous experimental studies demonstrated that drug exposure may induce not only resistance but also change other properties of tumor cells which may be related to tumor growth, invasiveness and immunogenicity [203, 247, 248, 169, 210]. Acquired drug resistance of leukemic cells due to pretreatment with cytostatic drugs influences the sensitivity of leukemic cells towards cytotoxic lymphocytes [249-254]. While some reports show decreased sensitivity of drug-resistant leukemic cells to cellular cytotoxicity [235, 236, 252, 253], Posovszky et al [254] reported that chemotherapeutic drugs including araC sensitize pre-B acute lymphoblastic leukemia (ALL) cells for CD95- and cytotoxic T-lymphocyte- mediated apoptosis. The present study shows that resistance of T-leukemic cells to araC is associated with increased sensitivity to NK cell-mediated lysis. These effects were not due to direct activity of araC on cell metabolism but rather to selection of cell population with altered susceptibility to NK cells since the resistant cells cultured for up to ten passages (about fifty

days) in a medium without the drug were lysed by NK cells to a greater extent than the parental cell population.

4.7 Mechanism of increased sensitivity of araC-resistant leukemic cells

Recent studies have shown that NK cells display cytolytic activities by engagement of receptors involved in NK cell activation and inhibition [60]. Although NK cells can kill target cells spontaneously without prior stimulation, a delicate balance between signaling through inhibitory (KIR, CD94-NKG2A) and activating receptors (NCRs-NKp30, NKp44, NKp46, NKG2D and DNAM-1) tightly regulates their activation [60]. Moreover, the relevance of the NKG2D/NKG2D ligand system for the immune surveillance in patient leukemia cells has been described [255]. Salih and co-workers [255] reported that leukemia cells from patients variously express MICA/B and ULBP. They also showed that patient leukemia cells were lysed by NK cells in an NKG2D-dependent fashion. The proposed role of the NKG2D receptor in innate and adaptive immune responses to cellular and tissue stress is based on the ability of the receptor to stimulate cytotoxic effects of NK cells and T cells against virally infected cells and tumor cells *in vitro* and *in vivo* [1]. Specifically, NKG2D receptor activation can induce target cell lysis and trigger the production of cytokines [42, 256] and chemokines [42, 257, 258], as well as perforin and granzyme involved in cellular lysis [259]. Based on these reports the role of specific ligands for NK cell activating receptors in the susceptibility of parental and araC-resistant leukemic cells to NK cell lysis was investigated. H9 and Molt-4 cells express some NKG2D ligands (particularly ULBP-2 and ULBP-3) in addition to PVR while they do not express other NKG2D ligands including MICA and MICB. This is in concordance with the hypothesis by Pende and coworkers [260] stating that most T-cell leukemia cell lines are characterized by a $MICA^{-}ULBP^{+}$ phenotype. The results in this study, with particular reference to the blocking experiments, show that increased expression of ULBP-2 and ULBP-3 rendered araC-resistant leukemic cell lines to become more sensitive

to NK cell lysis. Blocking PVR with mAb could not inhibit NK cell lysis, suggesting that DNAM-1 is not involved in NK cell lysis of H9 and Molt-4 cell lines. The increased NK cell lysis of araC-resistant leukemic cells was observed despite expression of MHC-I molecules on their surface. These findings suggest that ULBP-NKG2D interaction is a major determinant for susceptibility of H9 and Molt-4 cell lines to NK cell lysis. Recent experiments demonstrated that NKG2D/NKG2D ligand system stimulates immune surveillance of tumors [48, 261, 262]. The capacity of the NKG2D ligand-expressing tumor cell lines to stimulate tumor immunity *in vivo* was critically dependent on the expression levels of NKG2D ligands on the tumor cell surface. In another study NKG2D ligand surface expression led to increased susceptibility of malignant or virus-infected cells to NK cell-or T cell-mediated lysis [42, 256]. Since NK cell activity is guided by a balance of activating and inhibitory signals, and an enhanced NKG2D ligand expression is able to trigger NK cells overcoming inhibitory signals by MHC-I molecules [38, 42], even modest changes in NKG2D ligand expression may critically influence NK cell cytotoxic potential.

4.8 Mechanism of increased expression of NKG2D ligands in araC-resistant leukemic cells

Cell regulatory pathways such as ERK or AKT may influence the sensitivity of leukemic cells to araC [225-227]. Treatment of leukemic cells with inhibitors of AKT or ERK pathways was shown to increase their sensitivity to araC treatment [225-227]. Experiments performed in this study show both AKT and ERK to be constitutively activated in H9 cells, with increased activation of ERK1 (p44) but not AKT in resistant cells relative to their parental counterparts. Treatment of H9 cells with pharmacological inhibitors of ERK or AKT showed no toxicity to the cells and did not significantly influence activity of araC in resistant cells.

Gasser and co-workers [263] showed an induction of NKG2D ligand up-regulation by DNA damaging conditions such as ultraviolet light and the chemotherapy agents- cisplatin and araC- in fibroblasts and transformed ovarian epithelial cells from mice. NKG2D ligand expression was induced at the transcriptional level by mechanisms involving ataxia-telangiectasia-mutated (ATM) and ATM-and Rad-3-related (ATR) protein kinases [263]. Both AKT and ERK were also shown to increase the expression of NKG2D ligands and the sensitivity to NK cell-mediated lysis of transformed cells derived from solid tumors and leukemia [199, 228]. In fact, while Borchers et al [199] reported the involvement of ERK signaling in the induction of surface expression of NKG2D ligands in human tumor cells following H_2O_2-induced oxidative stress, Boissel et al [228] demonstrated that NKG2D ligand expression in chronic myeloid cells is regulated posttranscriptionally by BCR/ABL kinase through activation of PI3K and mammalian target of rapamycin (mTOR). The results presented in this study demonstrate that ERK but not AKT activation is associated with increased ULBP-2 and ULBP-3 expression resulting in enhanced sensitivity of araC-resistant leukemic cells to NK cell lysis.

5.0 Conclusion

In the course of this research, a highly sensitive coupled luminescent method (CLM) based on glyceraldehyde-3-phosphate dehydrogenase (G3PDH) release from injured target cells for the measurement of NK cell cytotoxicity was first established. In contrast to common methods like ^{51}Cr or Eu^{3+} release, CLM does not require the pre-treatment of target cells with labeling substances which could be toxic or radioactive. CLM provides a highly sensitive, safe, material saving (low E:T ratio is needed) and fast procedure for measurement of NK cell activity. CLM was then used to investigate the effect of HDACi including SAHA and VPA on the cytolytic activity of IL-2-activated NK cells and on the sensitivity of parental and araC-resistant leukemic cells to NK cell lysis.

SAHA and VPA at therapeutic relevant concentrations inhibited the cytotoxicity of NK cells against human leukemic cells. This inhibition was associated with decreased surface expression and function of NK cell activating receptors NKp30 and NKp46 as well as impaired granule exocytosis and inhibition of IFN-γ production. NFκB activation in IL-2-activated NK cells was inhibited by both HDACi. Pharmacologic inhibition of NFκB activity resulted in similar effects on NK cell activity like those observed for HDACi. These results demonstrate for the first time that HDACi prevent NK cytotoxicity by down-regulation of NK cell activating receptors probably through the inhibition of NFκB activation. VPA and other HDACi are being studied as potential treatment for leukemia and MDS and early reports suggest that they may have therapeutic effects in some forms of leukemia [87, 140, 141]. Moreover, vorinostat (SAHA) has recently been approved by the Food and Drug Administration for the treatment of CTCL [78]. On the other hand, VPA therapy was shown to be associated with the development of myelodysplastic changes in the marrow and acute

leukemia [264]. It has been proposed that VPA therapy may lead to secondary leukemia by increased DNA damage through chronic inhibition of HDAC [264]. It has also been demonstrated that in AML, NK cells express low levels of NCRs. The insufficiency of NCR-ligand interactions has been hypothesized as the underlying cause of the low susceptibility of leukemic blasts to lysis by autologous NK cells [265]. It may be speculated that VPA-induced down-regulation of NCRs may lead to deficient immune control by NK cells and thus contribute to leukemogenesis associated with VPA therapy. It should be noted that SAHA which is a more potent inhibitor of HDAC than VPA [87] was also more potent inhibitor of IFN-γ production, granule release, NFκB activation, NCR expression, and NK cell lytic activity against leukemic cells. More studies are required especially with treated patients to further elucidate the multifaceted roles of HDACi on NK cell activity.

Furthermore, it was demonstrated in this research, again for the first time, that T-cell leukemic cell lines that received araC treatment and became resistant to the drug are more sensitive to NK cell attack than their parental counterparts. This increased sensitivity was associated with a higher surface expression of ligands for the NK cell activating receptor NKG2D, notably ULBP-2 and ULBP-3 in araC-resistant H9 and Molt-4 cell lines. Blocking ULBP-2 and ULBP-3 or NKG2D with mAbs inhibited NK cell lysis. Constitutive activation of ERK but not AKT was higher in araC-resistant cell lines than in parental cell lines. Inhibition of ERK using ERK inhibitor PD98059 decreased both ULBP-2 and ULBP-3 expression and NK cell cytotoxicity. These results demonstrate that increased sensitivity of araC-resistant leukemic cells to NK cell lysis is due to higher NKG2D ligand expression, resulting from more active ERK signaling pathway.

It is possible that part of the efficacy of some chemotherapies and radiotherapies, most of which activate the DNA damage response [266, 267], is due to the induction of NKG2D

ligands which consequently enhances sensitivity of the cell to the immune system. This favors the insertion of an early autologous NK cell immunotherapy following therapeutic chemotherapy in patients suffering from hematological malignancies. Furthermore, the data presented for araC-resistant leukemic cells suggest that development of resistance to chemotherapeutic drugs may be associated with increased immunogenicity of tumor cells. Since such changes persist after cessation of treatment, it is of interest to show whether emergence of chemotherapy-resistant tumor clones with increased sensitivity to NK cell lysis also appear in patients who become refractory to common treatments with araC or other chemotherapeutic agents. The pathway leading to the up-regulation of NKG2D ligands looks promising in the search of targets for design of therapeutic agents to enhance the immunogenicity of transformed cells while reducing overall toxicity.

5.0.1 Zusammenfassung

Schnelle, empfindliche und material-sparende Methoden zur Messung der zytolytischen Aktivität natürlicher Killerzellen (NK), eine wichtige Determinante der NK Zell Funktion, sind entscheidend für die Analyse der physiologischen Funktion, als auch für die pathologisch veränderten Zustände des Immunsystems. Den Goldstandard für die Messung zellvermittelter Zytotoxizität stellt das Chromium (^{51}Cr) oder Europium (Eu^{3+}) release assay dar. Allerdings stellt die Messung der Zielzelllyse mittels dem ^{51}Cr oder Eu^{3+} release assay eine zeitaufwendige Methode dar und die notwendige Markierung der Zielzellen mit radioaktiven Substanzen bedeutet eine drastische Manipulation. Darüber hinaus kommt es aufgrund der vielen Zellen, die für den Test benötigt werden, zu hohen Hintergrundwerten. Verschiedene andere Methoden zur Messung von Zell Zytotoxizität sind widersprüchlich und weisen zahlreiche Mängel auf. Im Verlauf dieser Forschungsarbeit wurde zur Messung der zytolytischen Aktivität von Interleukin (IL)-2-aktivierten NK Zellen gegen die NK Zell sensitive erythroleukämische Zelllinie K562 erstmals die hoch empfindliche gekoppelte Lumineszenz Methode (coupled luminescent method (CLM)) etabliert. Diese Methode basiert auf der Messung der Glyceraldehyd-3-phosphat-dehydrogenasefreisetzung (G3PDH) aus lysierten Zielzellen. Zur Validierung der CLM wurden NK-resistente Neuroblastom (NB) Zellen verwendet. Alle getesteten NB Zelllinien (UKF-NB-2, UKF-NB-3, UKF-NB-4 and UKF-NB-$2^{r}VCR^{10}$) wurden durch IL-2 aktivierte NK Zellen lysiert. Im Gegensatz zu herkömmlichen Methoden wie ^{51}Cr oder Eu^{3+}, ist es bei der CLM nicht nötig eine Vorbehandlung der Zielzellen mit radioaktiven oder toxischen Substanzen vorzunehmen. CLM stellt daher eine hochsensible, sichere und materialsparende Methode zur Messung von NK Zellen dar.

Zunächst wurde CLM eingesetzt, um den Effekt von Histon-Deacetylase Inhibitoren (HDACi), Suberoylanilid Hydroxam Säure (SAHA) und Valproin Säure auf die zytolytische Aktivität IL-2-stimulierter NK Zellen und die Empfindlichkeit parentaler und Cytarabin (araC)-resistenter leukämischer Zellen auf NK Zelllyse zu untersuchen.

Die Verwendung von HDACi als Zytostatika zur Krebsbehandlung hat in letzter Zeit große Aufmerksamkeit errungen. Klinische Studien haben gezeigt, dass pharmakologisch relevante Mengen von HDACi wie SAHA oder VPA den Patienten sicher verabreicht werden können, und dass somit eine Behandlung von Krebs möglich ist. Die antitumorale Aktivität von HDACi liegt dabei in der direkten Wirkung auf Tumorzellen, indem diese die Hemmung der unkontrollierten Zellteilung bewirken, sowie Apoptose induzieren. Außerdem können HDACi das Tumorwachstum durch Inhibierung der Angiogenese und Zunahme der Immunogenität der Tumorzellen beeinflussen. Jedoch wurde noch nichts über den direkten Effekt von HDACi auf NK Zell Aktivierung beschrieben.

Im Rahmen dieser Forschungsarbeit wurde festgestellt, dass SAHA und VPA in therapeutisch relevanten Konzentrationen die Zytotoxizität von NK Zellen gegen Tumorzellen inhibieren. Diese Inhibierung war sowohl assoziiert mit einer Abnahme der Oberflächenexpression und Funktion der NK Zell aktivierenden Rezeptoren NKp30 und NKp46 (natürliche zytotoxische Rezeptoren, NCR) als auch mit einer beeinträchtigten granulären Exozytose und Inhibierung der Interferon Gamma (IFN-γ) Produktion. Die Nuclear factor kappa B (NFκB) Aktivierung in IL-2-aktivierten NK Zellen wurde durch beide HDACi inhibiert. Die pharmakologische Inhibierung der NFκB Aktivität zeigte ähnliche Effekte in der NK Zellaktivität wie mit HDACi. Diese Ergebnisse demonstrieren zum ersten Mal, dass HDACi über eine Herabregulierung von NK Zell aktivierenden Rezeptoren, vermutlich durch Inhibierung der NFκB Aktivierung, die NK Zytotoxizität erniedrigen.

VPA und andere HDACi werden als Behandlungsmöglichkeiten für Leukämien und Myelodysplastische Syndrome in Betracht gezogen und neueste Untersuchungen zeigen erste therapeutische Effekte bei bestimmten Leukämieformen. Darüber hinaus wurde kürzlich Vorinostat (SAHA) zur Therapie des kutanen T-Zelllymphoms (CTCL) zugelassen. Andererseits wurden unter VPA-Therapie myelodysplastische Veränderungen im Knochenmark und akute Leukämien beobachtet. Es wird vermutet, dass eine VPA-Therapie durch zunehmenden DNA Schaden aufgrund einer chronischen Inhibierung durch HDAC, zu einer sekundären Leukämie führen kann. Weiterhin wurde bewiesen, dass bei akuter myeloischer Leukämie (AML) NK Zellen eine geringe Expression von NCR zeigen. Die Insuffizienz der NCR-Liganden Interaktionen wird als Ursache für die erniedrigte Empfindlichkeit der leukämischen Blasten gegenüber der autologen NK Zell induzierten Lyse angenommen. Es kann angenommen werden, dass die VPA induzierte Herabregulierung von NCR zu einer defizienten Immunkontrolle durch NK Zellen führt und so zur Entstehung von Leukämien durch die VPA Therapie beitragen könnte. Von Interesse ist weiterhin die Tatsache, dass SAHA, ein weit potenterer Inhibitor von HDAC ist als VPA. SAHA ist ein stärkerer Inhibitor der INF-γ Produktion, der granulären Freisetzung, der NFκB Aktivierung, der NCR Expression und unterdrückt die lytische NK Zellaktivität gegen leukämische Zellen stärker. Es wird also erforderlich sein, besonders mit behandelten Patienten weitere Studien durchzuführen, um die vielfältigen Einflüsse der HDACi auf die NK Zellaktivität zu verstehen.

Zahlreiche experimentelle Studien haben belegt, dass die Medikamentengaben nicht nur Resistenzen induzieren, sondern auch andere Eigenschaften der Tumorzellen im Bezug auf Wachstum, invasives Verhalten und die Immunogenität verändern. Erworbene Resistenzen gegen Medikamente bei leukämischen Zellen, aufgrund einer Vorbehandlung mit Zytostatika, beeinflussen die Sensitivität der leukämischen Zellen gegenüber der zytotoxischen Aktivität

der NK Zellen. Während einige Veröffentlichungen eine Abnahme der Sensitivität von chemoresistenten Leukämie Zellen gegenüber der zellulären Zytotoxizität vorweisen, konnten Posovszky et al. (1999) zeigen, dass Chemotherapeutika wie araC, pre-B akute lymphoblastische Leukämie (ALL) - Zellen für CD95 - und zytotoxische T Lymphozyten vermittelte Apoptose sensibilisieren. Neuere Studien konnten zeigen, dass NK Zellen durch die Bindung von Rezeptoren zytolytische Aktivität entfalten, die in NK Zellaktivierung und Inhibierung involviert sind. NK Zellen sind in der Lage Zielzellen spontan und ohne vorausgegangenen Stimulus abzutöten. Darüber hinaus ist eine Aktivierung der NK Zellen über eine empfindliche Balance zwischen den transduzierten Signalen durch inhibitorische Rezeptoren (KIR, CD94-NKG2A) und aktivierende Rezeptoren (NCRs - NKp30, NKp44, NKp46, NKG2D und DNAM-1) reguliert. Zudem wurde die Bedeutung des NKG2D/NKG2D Liganden Systems als Überwachungsmarker für die Immunität von leukämischen Zellen beschrieben. Salih et al. (2003) berichteten, dass Leukämiezellen von Patienten verschiedenartiger Haupthistokompatibilitätskomplexe der Klasse I (MHC-I) verwandte A/B-Ketten (MICA/B) und UL16 Binding Protein (ULBP) exprimieren. Sie zeigten außerdem eine NKG2D abhängige NK-induzierte Zelllyse leukämischer Zellen.

In dieser Forschungsarbeit wurde wiederum erstmals gezeigt, das T Zell Leukämie Zelllinien, die zuvor mit araC behandelt wurden und eine Chemoresistenz erlangten, weitaus sensibler gegenüber den NK Zellen - Attacken waren, als parentale, unbehandelte Zellen. Diese Effekte sind nicht direkt der Wirkung von araC auf den Zellmetabolismus zuzuschreiben, sondern eher einer Selektion der Zellen, deren Empfänglichkeit für NK Zellen verändert ist. Die resistenten Zellkulturen, die bis zu zehn mal in medikamentenfreien Medium passagiert wurden, konnten durch die NK Zellen in einem größeren Ausmaß lysiert werden, als die parentale Zellpopulation.

Die erhöhte Sensitivität war assoziiert mit einer gesteigerten Oberflächenexpression der Liganden für die NK Zell aktivierenden Rezeptoren NKG2D, besonders ULBP-2 und ULBP-3 in araC-resistenten H9 und Molt-4 Zelllinien. Die Blockade von ULBP-2 und ULBP-3 oder NKG2D durch monoklonale Antikörper inhibierte die NK Zell-vermittelte Lyse. Die konstitutive Aktivierung der extrazellulären Signal-regulierten Kinase (ERK), aber nicht AKT, war höher in araC-resistenten Zelllinien als in parentalen Zelllinien. Eine Inhibierung von ERK durch den ERK Inhibitor PD98059 führte zu einer Abnahme der Expression von ULBP-2 und ULBP-3 und der NK Zell Zytotoxizität. Diese Ergebnisse deuten darauf hin, dass eine erhöhte Sensitivität von araC-resistenten leukämischen Zellen gegenüber einer NK Zell-vermittelten Lyse darauf zurückzuführen ist, dass die durch einen aktivierten ERK Signaltransduktionsweg ausgelöste Expression von NKG2D Liganden hochreguliert wird.

Möglicherweise ist die Induktion der NKG2D Liganden, die die Empfindlichkeit der Zellen gegenüber dem Immunsystem erhöht, Teil der Wirksamkeit von Chemo - und Radiotherapien. Dies favorisiert den Einsatz einer frühen autologen NK Zellimmunotherapie, anschließend an eine therapeutische Chemotherapie bei Patienten die an einer hämatologischen Krebserkrankung leiden. Weiterhin zeigen die Daten für araC-resistente leukämische Zellen, dass eine Resistenzentwicklung von Krebszellen gegenüber Chemotherapeutika, mit einer erhöhten Immunogenität der malignen Zellen einhergehen kann. Da diese Veränderungen nach Beendigung der Behandlung bestehen bleiben, ist es von Interesse aufzuzeigen, ob das Auftreten von chemotherapieresistenten Tumor Klonen mit erhöhter Sensitivität für NK Zell-vermittelte Lyse ebenfalls bei Patienten auftritt, die unempfindlich gegenüber bekannten Therapien mit araC oder anderen Chemotherapeutika geworden sind. Der Pathway, der zu einer Hochregulierung der NKG2D Liganden führt, ist viel versprechend für die Suche nach Zielen für das Design von therapeutischen Mitteln, um die Immunogenität von transformierten Zellen zu verstärken und die Toxizität zu reduzieren.

6.0 Literature

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7.0 Figure Legend

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8.0 Tables

9.0 Appendix

- Abbreviations
- Curriculum Vitae
- Publications
- Acknowledgement
- Declaration

Abbreviations

ADC	Analog to digital converters
ADCC	Antibody-dependent cellular cytotoxicity
ADP	Adenosine diphosphate
AML	Acute myeloid leukemia
APC	Allophycocyanin
APS	Ammoniumpersulphate
AraC	Cytosine arabinoside, cytarabine, 1-β-D-arabinofuranosylcytosine
AraCDP	araC diphosphate
AraCMP	araC monophosphate
AraCTP	araC triphosphate
ATCC	American type culture collection
ATM	Ataxia-telangiectasia-mutated
ATP	Adenosine triphosphate
ATR	ATM-and Rad-3-related
ATRA	All-trans retinoic acid
BCL-6	B cell lymphoma-6
BMT	Bone marrow transplantation
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
cDNA	Complementary DNA
CML	Chronic myeloid leukemia
CO_2	Carbondioxide
^{51}Cr	Chromium
Ct	Cycle-threshold
CTCL	Cutaneous T cell lymphoma
dCK	Deoxycytidine kinase
DC	Dendritic cells
DCSign	DC-specific intercellular molecule 3-grabbing nonintegrin
DEPC	Diethylpyrocarbonate
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNAM-1	DNAX accesorry molecule-1
DPG	Diphosphoglycerate
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
dUTP	Deoxyuridine triphosphate
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
eNOS	Endothelial nitric-oxide synthase
ERK	Extracellular signal-regulated kinase
Eu^{3+}	Europium
FACS	Fluorescent activated cell sorter
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FSC	Forward angle light scatter
g	Gram

g	Relative centrifugal force (RCF)
G3P	Glyceraldehyde-3-phosphate
G3PDH	Glyceraldehyde-3-phosphate dehydrogenase
GPI	Glycosylphosphatidylinositol
GPL	G3PDH/Phosphoglycerokinase/luciferase
GVL	Graft versus leukemia
h	Hour
H_2O	Water
H_2O_2	Hydrogen peroxide
HAT	Histone acetyltransferase
HCl	Hydrochloric acid
HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibitors
hENT1	Human equilibrative nucleoside transporter
HIF1	Hypoxia inducible factor 1
HLA	Human leucocyte antigen
IC	Inhibitory concentration
ICAM-1	Intercellular cell adhesion molecule-1
IFN	Interferon
IgG	Immunoglobulin
IL-2	Interleukin-2
IMDM	Iscove's modified dulbecco's medium
IRF	IFN regulatory factor
ITAM	Immunoreceptor tyrosine-based activating motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
kD	Kilo dalton
KIR	Killer inhibitory receptor
LDH	Lactate dehydrogenase
LFA-1	Leucocyte function antigen-1
LPS	Lipopolysaccharide
M	Molar
mAb	Monoclonal antibody
MACS	Magnetic associated cell sorting
MAPK	Mitogen activated protein kinase
MDR	Multidrug resistant
MDS	Myelodysplastic syndrome
$MgCl_2$	Magnesium chloride
MHC	Major histocompatibility complex
MICA	MHC class I chain-related molecule A
MICB	MHC class I chain-related molecule B
Min	Minute
ml	Milliliter
mM	Millimolar
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
NaCl	Sodium chloride
NAD^+	Nicotinamide adenine dinucleotide oxidized form
NADH	Nicotinamide adenine dinucleotide reduced form
$NaHCO_3$	Sodium hydrogen carbonate

NB	Neuroblastoma
NCAM	Neural cell adhesion molecule
NCR	Natural cytotoxicity receptor
NFκB	Nuclear factor kappa B
NK	Natural killer
NKG2A	Natural killer group 2A
NKG2D	Natural killer group 2D
NKp	Natural killer protein
nm	Nanometer
NO	Nitric oxide
NP-40	Nonidet 40
NT	Nucleoside transporter
PAGE	Polyacrylamide gel electrophoresis
PBMC	Polyclonal blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PG	Phosphoglycerate kinase
PI	Propidium iodide
PI3K	Phosphatidylinositol-3-kinase
PIM	Protease inhibitor mix
PMT	Photo multiplier tube
PVR	Polio virus receptor
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase polymerase chain reaction
SAHA	Suberoylanilide hydroxamic acid
SAP	Signaling lymphocyte activation molecule-associated protein
SB	Sodium butyrate
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SSC	Sideward scatter
STAT	Signal transducers and activator of transcription
TAP	Transporter associated with antigen processing
TBP2	Thioredoxin protein 2
TEMED	N,N,N',N'-Tetramethylethylenediamine
TNF	Tumor necrosis factor
TRAIL	Tumor necrosis factor related apoptosis inducing ligand
TSA	Trichostatin A
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick-end labeling
U	Unit
ULBP	UL16 binding protein
VCR	Vincristine
VEGF	Vascular endothelial growth factor
VPA	Valproic acid
vs	Versus
XLP	X-linked lymphoproliferative
°C	Degree celcius
µg	Microgram

µl	Microliter
µm	Micrometer
µM	Micromolar
%	Percent

Curriculum Vitae

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Educational Background

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Deutsch in Deutschland, Frankfurt, Germany	Jul 2000-Oct 2000
Volkshochschule, Hanau, Germany	Nov 1999-Jun 2000
University of Benin, Benin City, Nigeria	Oct 1991-Dec 1996

Edokpolo Grammar School, Benin City, Nigeria	Oct 1986-Sep 1991
Ebenezer Primary School, Benin City, Nigeria	Sep 1981-Jul 1986

Certificates obtained	**Date**
Diploma in Biology	Feb 2004
German language certificate (DSH)	Mar 2001
Bachelor of Science (B.Sc.) Microbiology	Dec 1996
Senior School Certificate	Sep 1991

Working Experience

Feb 2005 to date: Research assistant/Ph.D student at the laboratory of Prof. Dr. Jindrich Cinatl Jr., Hospital of Johann Wolfgang Goethe University, Frankfurt/Main, Germany with the aim of obtaining a doctorate degree in pharmaceutical technology. Topic: "Establishment and application of a highly sensitive coupled luminescent method (CLM) to study natural killer cell cytolytic activity".

June 2003 – Feb 2004: Diploma thesis (Topic: "Modulation of PSA-NCAM expression on neuroblastoma cells by valproate") at the urology research laboratory of Prof. Dr. Roman Blaheta, Clinic for Urology and Pediatric Urology, Hospital of Johann Wolfgang Goethe University, Frankfurt, Germany. Here I mastered several cell culture techniques, flow

cytometry, as well as molecular biology working methods.

May 1998 – October 1998: Laboratory scientist at the pharmaceutical company SCIO Nigeria Ltd, Benin City, Nigeria.

June 1997 - April 1998: National Youth Service Corps (NYSC) at National Oil and Chemical Marketing Plc., Lagos, Nigeria. I was involved in the sales and marketing of chemical products like bitumen.

April 1993 – July 1993: Industrial attachment at the medical laboratory of Nigerian National Petroleum Corporation staff clinic, Benin City, Nigeria.

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- Kleinschmidt MC, Michaelis M, **Ogbomo H**, Doerr HW, Cinatl J Jr. Inhibition of apoptosis prevents West Nile virus induced cell death. BMC Microbiol. 2007; 7:49.

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Declaration

I hereby declare that I independently performed and wrote this dissertation and used no literature or resource other than those indicated.

Frankfurt am Main, January 2008 Henry Ogbomo