



MASTERARBEIT

„Effects of DNA Methyltransferase- and Histone Deacetylase Inhibitors on
ALK Positive Anaplastic Large Cell Lymphoma“

„Auswirkung von DNA Methyltransferase- und Histondeacetylase Inhibitoren
auf das ALK positive anaplastische großzellige Lymphom“

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Index

1. Abstract.....	5
2. Zusammenfassung	6
3. Introduction.....	8
3.1 Epigenetics – Modern Definition	8
3.2 DNA –Methylation	8
3.2.1 Mammalian DNA methyltransferases.....	8
3.3 Histone Modifications.....	9
3.4 Epigenetics and cancer	10
3.4.1 DNA methylation in cancer	10
3.4.2 Histone modifications in cancer	10
3.5 Anaplastic large cell lymphoma	12
3.5.1 ALK deregulation through the NPM-ALK fusion protein	12
3.5.2 NPM–ALK signaling pathways.....	13
3.5.3 ALK positive ALCL and Epigenetic Silencing	16
3.6 Epigenetic drugs and cancer therapy	16
4. Material and Methods	18
4.1 In vitro studies- Working with cell cultures	18
4.2 In vivo studies-Working with Mice.....	19
4.3 Cell Cycle Analysis by Propidium Iodide (PI) Staining	21

4.4 Work with DNA	21
4.4.1 Isolation of genomic DNA	21
4.4.2 Bisulfite Conversion and methylated DNA analysis by COBRA.....	22
4.4.3 Methylation-sensitive high resolution melting (MS-HRM)	23
4.5 Work with Proteins.....	24
4.5.1 Western Blot/Protein Extraction	24
4.5.2 Isolation of Core Histones.....	26
4.6 Immunohistochemistry	26
4.7 Antibodies	28
5. Results.....	29
5.1 <i>In vitro</i> and <i>in vivo</i> analysis after treatment with Aza	29
5.1.1 Effects of Aza on ALK positive ALCL and ALK negative ALCL cell lines	29
5.1.2 Effects of Aza in a Xenograft model.....	30
5.1.3 FACS analysis of Aza treated mice.....	32
5.1.4 DNA methylation status of tumor suppressor genes	33
5.1.5 Protein analysis of Aza treated Xenografts	36
5.1.6 Immunohistochemistry staining of Aza treated tumor tissues	37
5.2 <i>In vitro</i> and <i>in vivo</i> analysis after treatment with MS-275.....	39

5.2.1 Effects of MS-275 on ALK positive ALCL and ALK negative ALCL cell lines.....	39
5.2.2 In vitro protein analysis of MS-275 treated cell lines.....	40
5.2.3 <i>In vivo</i> analysis of Xenograft mice treated with MS-275	40
5.2.4 FACS analysis of MS-275 treated mice	41
5.2.5 Protein analysis of MS-275 treated Xenografts.....	42
5.2.6 Immunohistochemistry staining of MS-275 treated mice tissues	43
6. Discussion	45
7. Supplementary.....	48
7.1 Aza treatment	48
7.1.1 Xenograft 1	48
7.1.2 Xenograft 2	51
7.1.3 Xenograft 3	53
7.2 MS-275 treatment.....	54
7.2.1 Xenograft 1	54
8. Abbreviations	55
9. References	58
10. Appendix.....	67
CURRICULUM VITAE	67
Acknowledgements.....	68

1. Abstract

Epigenetics studies heritable changes in gene expression patterns, which are dependent on processes such as DNA methylation, histone modifications or RNA directed silencing mechanisms. Epigenetic therapy seems to be effective for treatment of such reversible modifications and is promising for cancer therapy.

Anaplastic large cell lymphoma (ALCL) is a non-Hodkin lymphoma of T-cell origin. In 80% of cases, a chromosomal translocation (t 2;5) occurs, which generates the oncogenic fusion protein NPM-ALK. The fusion protein acts as a constitutive active kinase and is involved in important signaling pathways, such as JAK/STAT, PI3K and MAPK, which play a role in controlling cell proliferation and survival.

Our study aimed at studying epigenetic drugs for the treatment of ALCL. We have tested two promising drugs, namely 5-Aza-2'-deoxycytidine (Aza), a DNA methyltransferase inhibitor and MS-275, a histone deacetylase inhibitor, *in vitro* using human ALCL cell lines and *in vivo* in a Xenograft model .

Treatment with Aza using the three ALCL cell lines Karpas-299 (ALK positive), SR-786 (ALK positive) and Mac-2A (ALK negative), showed decreased cell number and survival, increased apoptosis and decreased methylation patterns of selected target genes. *In vivo* studies in NOD/SCID mice using the human ALK positive cell line Karpas-299 showed decreased or no tumor growth, increased apoptosis and lower cell proliferation upon treatment with Aza. As a consequence of this, we observed decreased promoter DNA methylation and reduced DNMT1 protein levels in treated mice.

In vitro treatment with MS-275 in the ALCL cell lines resulted in decreased cell number and survival. ALK positive ALCL cell lines showed lower expression of NPM-ALK after 4 days of treatment. However, *in vivo* Xenograft experiments with MS-275 showed less pronounced effects including a slight growth reduction, but no weight reduction compared to control mice, although acetylation of histones was slightly increased in treated mice.

Based on these findings, Aza showed promising results in the treatment of ALK positive ALCL both *in vitro* and *in vivo*, whereas the use of MS-275 *in vitro* showed inhibition of cancer cell growth, but results could not be repeated *in vivo*.

In conclusion, targeting epigenetic enzymes like DNMTs and HDACs could provide a possible option for therapy in this disease.

2. Zusammenfassung

Epigenetische Modifikationen sind vererbbar und können Genexpressionmuster bei verschiedenen Krebszelllinien verändern. Diese Veränderungen beinhalten Prozesse wie DNA Methylierung, Histonmodifikationen oder RNA-abhängige Mechanismen. Die Behandlung von solchen reversiblen Modifikationen könnte eine effektive Therapie in malignen Tumoren darstellen.

Das Großzellige-anaplastische Lymphom (ALCL) zählt zu den nicht Hodgkin T-Zell Lymphomen. Die chromosomale Translokation (t 2;5) generiert das onkogene Fusionsprotein NPM-ALK und ist in 80% aller ALCL-betroffenen Personen vorhanden. Dieses Fusionsprotein kontrolliert verschiedene Signaltransduktionswege wie JAK/STAT, PI3K und MAPK und spielt eine wichtige Rolle bei der Kontrolle von Zellproliferation und Überleben.

Die Entwicklung neuer, zukünftiger Therapien für die Behandlung von ALCL und die Forschung im Bereich der epigenetischen Arzneimittel waren die Ziele unserer Arbeit. Im Zuge unserer Forschung testeten wir die zwei erfolgversprechenden Arzneimittel, 5-Aza-2-deoxycytidine (Aza), ein DNA-Methyltransferase Inhibitor, und MS-275, ein Histondeacetylase Inhibitor, sowohl *in vitro* als auch *in vivo*.

Die Behandlung mit Aza führte in allen drei getesteten ALCL Zelllinien zu verminderter Zellzahl, erhöhter Apoptose und verminderter Methylierung von Tumorsuppressorgenen.

In vivo wurde ein Xenograft Experiment durchgeführt wobei NOD/SCID Mäuse und die ALK positive Zelllinie Karpas-299 verwendet wurden. Nach der Behandlung konnten wir eine deutliche Verminderung der Tumorgroße feststellen, in einigen Fällen waren die Mäuse sogar tumorfrei. Das Tumorgewebe aus den behandelten Mäusen war stark apoptotisch und die Zellproliferation war vermindert. Ebenso beobachteten wir einen allgemeinen Rückgang der DNA-Methylierung, sowie ein verringertes Proteinlevel von DNMT1 in den behandelten Mäusen.

Die *in vitro* Behandlung mit MS-275 von ALCL Zelllinien führte zu reduzierter Zellzahl und vermindertem Zellüberleben. Die ALK positiven Zelllinien zeigten niedrigere NPM-ALK Proteinexpression nach viertägiger Behandlung mit MS-275. *In vivo* konnte allerdings nur eine leichte Reduktion der Tumorgroße, aber keine Reduktion von Tumorgewicht, beobachtet werden, obwohl die Acetylierung in Tumoren nach Behandlung mit MS-275 etwas erhöht war.

Diese Ergebnisse zeigen, dass Aza ein vielversprechendes Therapeutikum in ALK positiven ALCL darstellt, während MS-275 nur einen Effekt *in vitro*, aber nicht im Xenograft Modell zeigte.

Zusammenfassend kann gesagt werden, dass Medikamente, die eine Rolle bei der Regulierung von epigenetischen Enzymen haben, möglicherweise als zukünftige Behandlungsmethode in Betracht gezogen werden können.

3. Introduction

3.1 Epigenetics – Modern Definition

Epigenetics is the study of heritable changes in gene expression, which are not encoded in the sequence of the genome. In contrast to genetic changes that are irreversible, epigenetic changes are reversible (1). There are three main epigenetic mechanisms: RNA directed silencing, posttranslational histone modifications and DNA methylation (2) of which the latter two will be discussed below.

3.2 DNA –Methylation

DNA methylation in mammals occurs at the cytosine residue of CpG dinucleotides and leads to heritable silencing of genes and non-coding genomic regions by either preventing access or recruiting regulatory proteins to DNA (3).

Methylated CpG dinucleotides are prevalent in heterochromatic regions, such as repetitive sequences, centromeric repeats and retrotransposon elements. On the other hand CpG-rich DNA stretches called “CpG islands” (2), which are found in approximately 60% of promoters of mammalian genes, are usually unmethylated and the corresponding gene is not silenced (3).

A reason why DNA is heavily methylated at repetitive sequences might be to repress i.e. parasitic DNA sequences, which are foreign sequences incorporated into the host DNA (4). This defensive role of DNA methylation is also important for the genomic stability for silencing non-coding and transposable DNA elements (5). Thus, DNA methylation might have evolved as a defensive mechanism against foreign invading DNA.

3.2.1 Mammalian DNA methyltransferases

The enzymes that catalyze the transfer from S-Adenosylmethionine (SAM) to DNA are called DNA methyltransferases. There are *de novo* and *maintenance* methyltransferases. *De novo* methyltransferases can target unmethylated DNA, whereas maintenance methyltransferases show a preference for hemimethylated DNA.

In mammals there are five reported members of the DNMT family: DNMT1, DNMT2,

DNMT3a, DNMT3b, and DNMT3L (3).

De novo methyltransferases DNMT3A and DNMT3B can work independently from S-phase and establish methylation patterns during embryonic development. They are highly expressed in embryonic stem (ES) cells and to a lesser extent in differentiated cells (6).

The maintenance methyltransferase DNMT1 works mainly on hemimethylated DNA during replication, but it has been recently reported to also have *de novo* activity (3). DNMT2 contains only the conserved catalytic domain and was suggested to be involved in the methylation of tRNAs and silencing of transposons in flies (3,7). DNMT3L has no DNMT activity but is involved in genomic imprinting processes by interacting with DNMT3a/3b (3).

3.3 Histone Modifications

There are two main forms of chromatin: the decondensed and early replicating euchromatin and the inaccessible, gene poor and late replicating heterochromatin. Chromatin is organized in nucleosome particles of a histone octamer around which 147 bp of DNA is wrapped (8).

The protein octamer is build of two molecules of each canonical histone protein H2A, H2B, H3 and H4. Histones are small, basic proteins. They contain a globular C-terminal domain and a flexible N-terminal tail (9). Post-translational modifications usually occur at the histone tail, although the globular domains can also undergo such modifications. Different chemical groups, such as methyl-, acetyl- and phosphate-groups, and different degrees of methylation like mono-, di- and tri-methylation are involved in these types of modifications (8). There are also other modifications that have recently been identified, such as ubiquitination, sumoylation, ADP-ribosylation, biotinylation and proline isomerisation (10-12). Important enzymes involved in histone modifications are histone acetyltransferases (HATs), which are targeting lysine residues by adding acetyl groups, and histone deacetylases (HDACs), which are involved in removing these groups (13). Serine or threonine residues can be targeted by kinases, which add phosphate groups, while phosphatases (PPTases) work on removing these groups. Methyltransferases can interact with lysine or arginine residues by adding methyl groups (HKMT, histone lysine methyltransferases) (13) or PRMT, (protein arginine methyltransferases) and

histone demethylases such as JmjC and LSD1 catalyse the reversible process (14,15).

In the biological context, histone tail modifications are involved in chromatin packaging activate or repress transcription depending on the modification and the residues that are modified. In general, acetylation is correlated with transcriptional activation (16), whereas methylation leads either to activation or repression. For example, an active gene promoter usually has trimethylation of lysine 4 on histone H3 (H3K4me3) (17), whereas marks such as trimethylation of H3K9 (H3K9me3) and H3K27 (H3K27me3) are repressive marks correlated with transcriptional repression (11).

3.4 Epigenetics and cancer

There are several epigenetic characteristics that distinguish normal from cancer cells. These include global DNA methylation changes, histone modification patterns and chromatin-modifying enzyme expression profiles (18,19), which all have an influence on the initiation and progression of cancer.

3.4.1 DNA methylation in cancer

Changes in the CpG methylation status in the cancer epigenome include global hypomethylation of the cancer genome and local hypermethylation of CpG island promoters including tumor suppressor genes (8). Binding of chemical carcinogens and spreading of the UV-induced mutations are also increased by methylation of CpG sites (8).

Repetitive sequences in cancer are usually hypomethylated, which leads to chromosomal instability such as translocations, gene disruption and reactivation of endoparasitic sequences (20,21). Hypermethylation involves genes that are part of important cellular pathways such as DNA repair, cell cycle control and apoptosis (22,23).

3.4.2 Histone modifications in cancer

Alterations of histone modifications are also correlated with the cancer phenotype. HDACs are involved in the hypoacetylation process and have been found to be overexpressed (24) or mutated (19). An overall reduction of acetylation of H4K16 has

been reported for breast and liver cancer (25). There is also a global loss of the active H3K4me3 (26) and the repressive H4K20me3 mark (27) and increased values of the repressive marks H3K9me3 (28) and H3K27me3 (29). These modifications attribute to aberrant expression of both histone methyltransferases such as EZH2 and NSD1 (30,31) and histone demethylases such as GASC1, LSD1, JmJc and others(32-34).

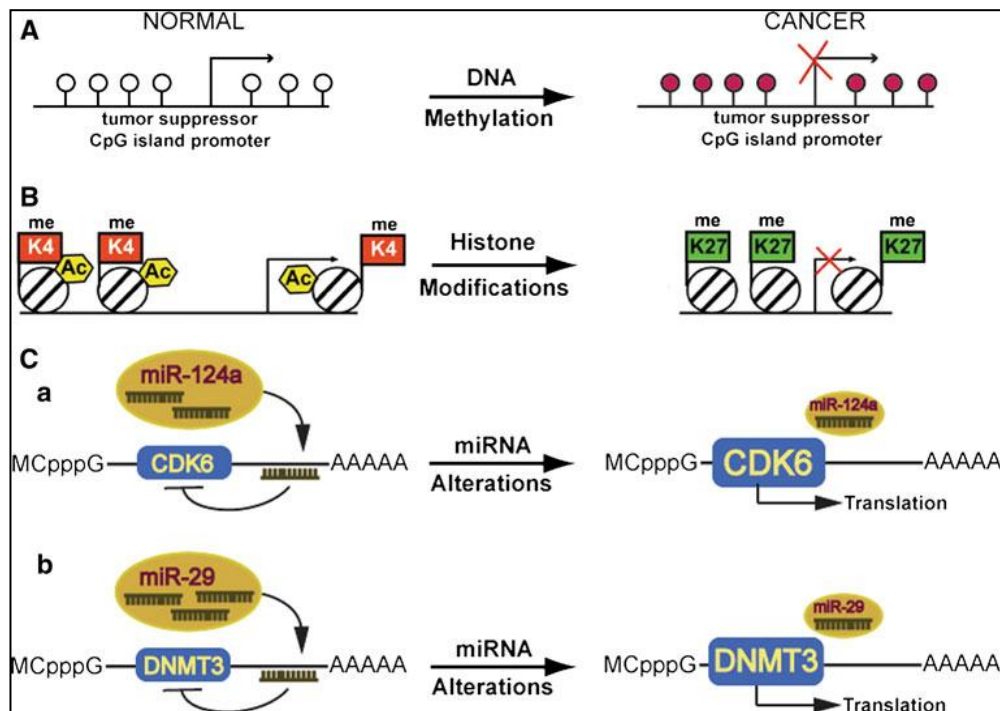


Figure 1 Epigenetic alterations during oncogenesis. A DNA methylation alterations in cancer. In normal cells, CpG island promoters are generally unmethylated (white circles) and active, as in the case of tumor suppressor genes. An active gene shows an open chromatin structure with no nucleosome upstream of the transcription start site (black arrow). During tumorigenesis, tumor suppressor gene promoters with CpG islands become methylated (red circles) resulting in the formation of silent chromatin structure and aberrant silencing (red X on the black arrow). B In normal cells active tumor suppressor genes (black arrow) show an enrichment of active histone marks such as acetylation (yellow polygon) and K4 methylation (red rectangular) of histone H3. In cancer cells, gene silencing of tumor suppressors may occur through the enrichment of repressive histone marks like H3K27 methylation (green rectangular) mediated by the PRC complex and the removal of active histone marks (acetylation and H3K4 methylation). C Abnormal miRNA expression in cancer. a Epigenetic changes like DNA methylation and histone medications are responsible for aberrant miRNA expression. For example, miR-124a is under epigenetic control in acute lymphoblastic leukemia (ALL) due to the fact that is embedded in CpG island regions that are silenced by hypermethylation. The result is that miR-124a is downregulated in ALL (when compared to normal cells) and its target CDK6 is up-regulated. b Epi-miRNAs are regulators of epigenetic effectors. For example, miR-29 family targets DNMT3a and DNMT3b. In normal cells, the levels of miR-29 are increased leading to decreased expression of its targets (DNMT3a and DNMT3b), normal patterns of DNA methylation and expression of tumor suppressor genes. In lung cancer cells, miR-29 is down-regulated resulting in up-regulation of DNMTs and hypermethylation and silencing of tumor suppressor genes

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3.5 Anaplastic large cell lymphoma

Anaplastic large cell lymphoma is classified into the group of T-cell non-Hodgkin Lymphomas (NHL). It was first identified in 1985 by Stein et al (35) as a large cell lymphoma with anaplastic cytology, uncommon growth sinus patterns, and high expression of the antigen CD30.

In 80 % of cases (36), there is a translocation $t(2;5)$, which was first described in the early 1990s by Morris and colleagues (37). It involves the receptor tyrosine kinase anaplastic lymphoma kinase (ALK) on 2p23 and nucleophosmin (NPM1) on 5q35.

There are three distinct T-cell ALCL tumor classes: ALK positive ALCL, ALK negative ALCL (listed in the same category) and primary cutaneous (C-ALCL), which might be a different entity (38). The histology of ALK positive ALCL and ALK negative ALCL is quite similar. They form cohesive clusters of pleomorphic large cells with high mitosis rate and show the CD4+ and CD30+ phenotype (39). The large kidney shaped cells are “hallmark cells” for this type of tumor, although some of the ALK positive ALCL tumors can also contain small cells and lymphohistocystic variants (10-20% of cases from ALCL) (40).

Diagnosis of ALK negative ALCL is featured on its strong expression of CD30 (41). Cutaneous ALCL, a CD4+ T-cell tumor, consists of large cohesive anaplastic cells with irregular nuclei that show similarity with those seen in systemic ALCL (42). Primary ALCL usually manifests during childhood. Current treatment of ALCL is based on the use of a combination of chemotherapy and CD30-agonist antibodies such as SGN-30 (43). ALK positive ALCLs have a better treatment prognosis than ALK negative ALCL or other T-cell NHLs (44).

3.5.1 ALK deregulation through the NPM-ALK fusion protein

Anaplastic lymphoma kinase belongs to the transmembrane tyrosine receptor kinases (RTK) and is a member of the insulin receptor superfamily. It is activated by phosphorylation of its YXXXY tyrosine motif. Autophosphorylation of this motif leads to a constitutively active NPM-ALK protein, which can cause oncogenic transformation of cells (45).

The expression of the wild type ALK is usually limited to the central and peripheral nervous system and a role for midgut and neural tissue development has been

demonstrated (46-48).

The ALK fusion partner NPM1 is a carrier protein involved in the transport of newly synthesized proteins between the cytoplasm and the nucleolus, in the regulation of cell division, in DNA repair, transcription and genomic stability (49).

3.5.2 NPM–ALK signaling pathways

Signaling through NPM-ALK changes several cellular processes. The constitutive active kinase activates pathways such as: JAK/STAT, PI3-K/AKT, MAPK and PLC γ (50), leading to proliferation, prolonged tumor cell survival, cytoskeletal rearrangements and cell migration.

JAK/STAT signaling contributes to regulatory functions of cytokins and controls processes such as cell growth, differentiation and survival. Phosphorylated JAK activates STATs and controls the mitogenic and antiapoptotic effects of ALK (51,52). STAT3 is phosphorylated and directly activated by JAK3 and its activation leads to translocation of STAT3 into the nucleus and increases the transcriptional levels of antiapoptotic molecules (53-58). STAT3 is also involved in the regulation of MYC, a potent oncogenic transcription factor, which is elevated in NPM-ALK transfected rat fibroblasts (59). Inhibition of STAT3 in ALK positive ALCL leads to apoptosis and cell cycle arrest (59). STAT5B is also activated by NPM-ALK via JAK2 and has a function in cell growth promotion (52,60), whereas STAT5A and SHP1 are epigenetically silenced via DNA methylation (61,62).

The PI3-K/AKT pathway is involved in the regulation of several pathways such as the mTOR pathway, cyclin dependent kinase (CDK) inhibitors, p21 and p27, cyclin D, p53, and plays a role in different cellular processes like cell growth, and apoptosis (63). PI3-K/AKT also induces the Sonic Hedgehog signaling pathway (SHH). Sonic Hedgehog regulates T-cell differentiation, T-cell receptor repertoire selection, activation of the peripheral T-cell and inhibition of apoptosis in germinal center of the B-cells (50). Sonic Hedgehog is expressed in 100 % of the ALK positive ALCL and 85% of ALK negative ALCL (64). It was suggested that inhibition of PI3-K/AKT/SHH pathway can increase the cell viability and can generate cell cycle arrest in ALK positive ALCL cell lines (64).

The MAPK pathway activated by NPM-ALK is important for cell growth and differentiation. NPM-ALK has a role as docking molecule and activates the RAS-ERK

pathway through docking different proteins such as insulin receptor substrate-1, SRC, SHC and PLC γ . Members of the ALK family such as stress activated protein kinase/C-JUN, N-terminal kinase and can be activated by environmental stress, inflammatory cytokines, and growth factors. They play a role in cell growth and survival, and have been also related to oncogenesis (50).

The WNT/ β -catenin pathway is involved in regulating cell fate decisions during differentiation and development. Accumulation of β -catenin in the cytoplasm and its translocation to the nucleus leads to binding and activation of members of the (TCF/LEF) family and upregulation of oncogenic proteins such as MYC and CYCLIN D1 (50). T-cell lymphoma such ALK positive and ALK negative ALCL are TCF-1/LEF1 negative and a loss of PTEN expression (tumor suppressor–phosphatase and tumor homolog) is found in 66.7% of all ALCL cases (65,66).

NOTCH is also involved in ALCL signaling and has a role in thymic maturation, peripheral T-cell proliferation and survival (67). Approximately 50% of T-ALLs have mutations in NOTCH and it was also found that NOTCH and Jagged (ligand of NOTCH) are highly expressed in ALK positive and ALK negative ALCLs (68).

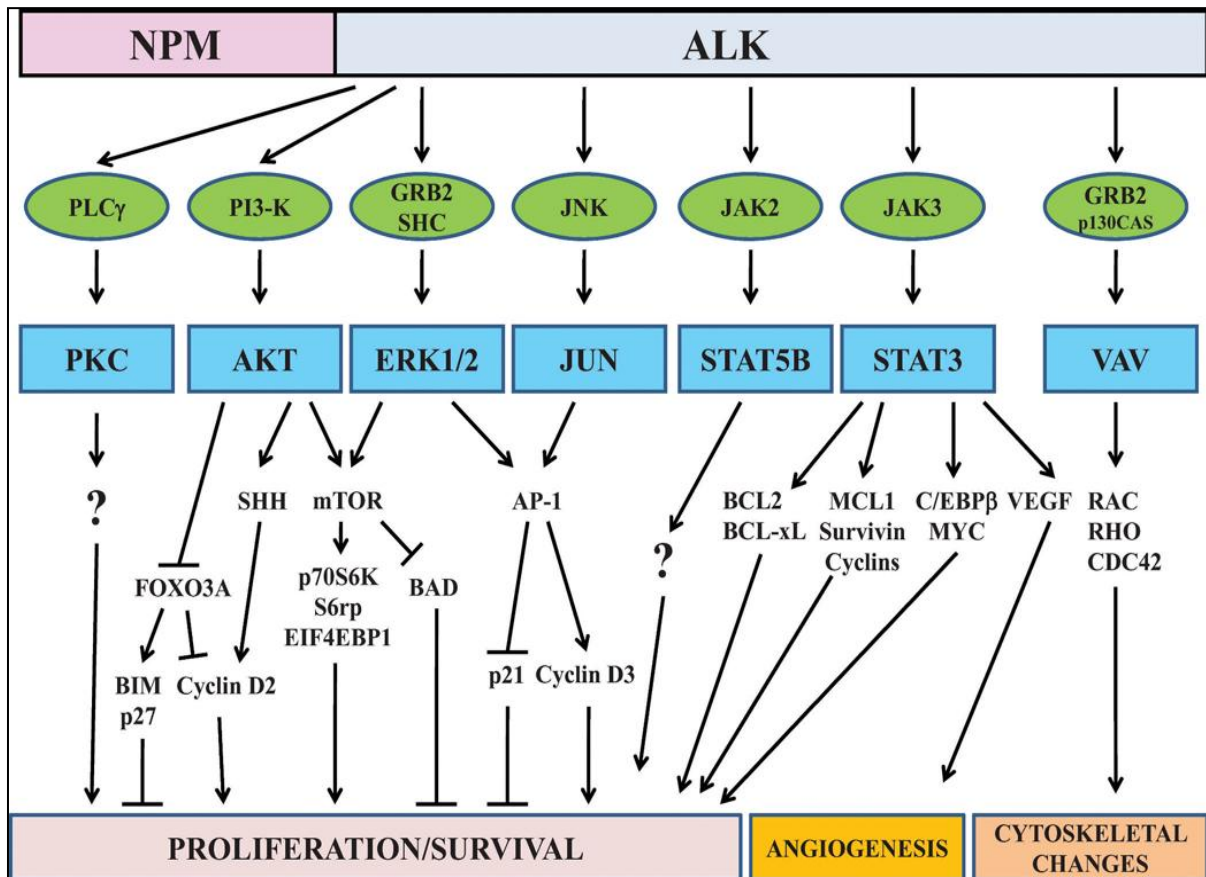


Figure 2 Summary of NPM-ALK signaling network. Constitutive NPM-ALK signaling in anaplastic large cell lymphoma (ALCL) exerts its oncogenic effects via a complex network of redundant and interacting pathways. The MAPKs (ERK1/2 and JNK) have a central role in driving proliferation/survival via the induction of the AP-1 transcription factors and subsequent down-regulation of p21 (inhibitor of cell cycle progression), and upregulation of cyclin D3. Both ERK1/2 and PI3-K/AKT activate mTOR, which down-regulates the proapoptotic protein BAD and phosphorylates proteins p70S6K, S6RP, and EIF4E-BP1, thereby enabling messenger RNA translation and protein synthesis to proceed. The PI3-K/AKT pathway further enhances proliferation by increasing the expression of cyclin D2 via signaling through SHH and FOXO3A inhibition. FOXO3A inhibition also decreases the expression of the proapoptotic protein BIM and the negative cell cycle regulator p27. STAT3 is another major regulator of ALCL cell proliferation/survival via its upregulation of cyclins, MYC, C/EBPb, BCL2, MCL1, BCL-xL, and survivin. STAT3 signaling also promotes tumor growth/survival by stimulating the expression of the proangiogenic VEGF. STAT5B and the PLCg/PKC pathway are activated by NPM-ALK and promote cell proliferation/survival growth, although the downstream mediators are unclear. Finally, the anaplastic morphology of ALCL is a result of various cytoskeletal changes that may be mediated by signaling through GRB2/p130CAS/VAV and the RHO GTPases (RAC/RHO/CDC42). NPM-ALK is not drawn to scale. Abbreviations: ALK, anaplastic lymphoma kinase; AP-1, activator protein 1; BAD, BCL2 agonist of cell death; BCL2, B-cell lymphoma 2; BCL-xL, B-cell lymphoma-extra large; BIM, BCL2-like 11; C/EBPb, CCAAT/enhancer-binding protein b; EIF4E-BP1, eukaryotic translation initiation factor 4E-binding protein 1; ERK1/2, extracellular signal-regulated kinases 1/2; FOXO3A, forkhead box O3A; GRB2, growth factor receptor-bound protein 2; JAK2, Janus kinase 2; JAK3, Janus kinase 3; JNK, c-JUN N-terminal kinase; MAPKs, mitogen-activated protein kinases; MCL1, myeloid cell leukemia sequence 1; mTOR, mammalian target of rapamycin; MYC, myelocytomatosis oncogene; NPM, nucleophosmin; PI3-K, phosphatidylinositol-3 kinase; PKC, protein kinase C; PLCg, phospholipase C g; p70S6K, p70S6 kinase; p130CAS, p130CRK associated substrate; SHC, SRC-homology-2-domain-containing transforming protein; SHH, sonic hedgehog; STAT3; signal transducer and activator of transcription 3; STAT5B; signal transducer and activator of transcription 5B; S6RP, S6 ribosomal protein; VEGF, vascular endothelial growth factor.

From Anaplastic Large Cell Lymphoma -Kinney et al

3.5.3 ALK positive ALCL and Epigenetic Silencing

Aberrant DNA methylation during oncogenesis contributes to malignant transformation. In ALCL aberrant promoter methylation has been detected for some candidate tumor suppressor genes such as p16 (INK4a), NFATC and SHP1 (69-71). Furthermore, it has been observed that STAT3 has an influence on epigenetic silencing, by inducing the expression of DNMT1. This leads to hypermethylation of SHP1 and thereby inhibits the ubiquitin-dependent degradation of NPM-ALK (71-73). The transcription factor NFATC is involved in the regulation of proliferation and proapoptotic effects. NFATC has been found to be silenced in lymphomas by hypermethylation of its promoter (69).

Aside from this, a candidate gene approach looking at different commonly hypermethylated genes in cancer revealed high levels of methylation in ALCL cell lines and patient samples (Hassler et al. unpublished results).

3.6 Epigenetic drugs and cancer therapy

Epigenetic changes are reversible and this is used for developing effective anticancer therapy, as epigenetically silenced genes can be reactivated by treatment with DNA demethylation drugs and/or histone deacetylase inhibitors. DNA methylation inhibitors such as Aza and Zebularine or histone deacetylase inhibitors including SAHA (Suberoylanilide hydroxamic acid), valproic acid (VPA) and trichostatin A (TSA) show antitumor activity. Aza is FDA approved for treating myelodysplastic syndrome in patients (74-76). SAHA is approved for the treatment of cutaneous T-cell lymphoma (77).

Nucleoside analogs such as Aza, which are DNA methyltransferase inhibitors, are incorporated into DNA forming covalent complexes with DNA methyltransferases (78-80). These inhibitors are only incorporated when the cell is actively dividing, as it is the case for a cancer cell. The current idea is that the inhibitors reactivate silenced tumor suppressor genes. However if the dosage is too high, side effects such as cytotoxicity can occur (81). It was also shown that vessel formation, which is important for tumor formation, is decreased by using Aza and Zebularine (82).

HDAC inhibitors are the second class of epigenetic drugs used for cancer therapy. They show anti-proliferative activity in cancer cells probably by activation of the G2

checkpoint, which in normal cells leads to cell cycle arrest, while in cancer cells this pathway is defective (83).

The HDAC inhibitor used for this study, MS-275, is a synthetic benzamide derivate, which has already been tested in clinical trials with patients with advanced solid tumors or lymphomas (84), where it could be shown that H3/H4 acetylation, p21 expression and Caspase 3 activation were induced after treatment in bone marrow mononuclear cells, however, patients response was relatively poor (85).

Furthermore, in preclinical studies, it was suggested that the combination of HDAC and DNMT inhibitors might show anti-tumor effects and re-expression of epigenetically silenced tumor suppressor genes (86). Studies were conducted in patients with advanced non small cell lung carcinoma (NSCLC). It was found that, the doses used were well tolerated and two of ten patients showed stable disease status. Therefore, the combination of the inhibitors might also be a possible treatment strategy to reactivate epigenetically silenced genes (86).

4. Material and Methods

4.1 In vitro studies- Working with cell cultures

Chemicals and reagents

5-Aza-2'-deoxycytidine (Sigma-Aldrich) was dissolved in PBS (GIBCO) to a concentration of 100 mM. Then it was diluted to 1 mM and stored at -80°C.

MS-275 was obtained from (Selleck Chemicals LLC) and diluted with DMSO (Sigma–Aldrich) to a final concentration of 1mM and also stored at -80°C.

Cell culture

Mac-2A and Karpas-299 (ALK positive ALCL) and SR-786 (ALK negative ALCL) human cell lines were obtained from collaborators. Cells were grown in RPMI 1640 medium (GIBCO) containing 10 % FBS (fetal bovine serum) and 1 % penicillin/streptomycin. Cells were grown at 37°C in an atmosphere of 5 % CO₂ and 95 % room air.

Mac-2A, Karpas-299 and SR-786 were seeded in six well plates (BD Falcon™) at a density of 0.5×10^6 cells per ml in RPMI medium (2 ml). Four of the six wells were treated with 2 µl of 1 mM 5-aza-2'-deoxycytidine (final concentration 1 µM). Two wells were used as control and treated with 2 µl of PBS. 5-aza-2'-deoxycytidine was added only once at the beginning of the treatment.

For MS-275 treatment, cells were seeded as described above and treated with 1 µl of 1 mM MS-275 (final concentration 0.5 µM). It was added over a period of 4 days to each well/each day. Two control wells were treated with an equal volume of DMSO.

1 ml of RPMI medium was added every second day to each well. Cells were counted using a C-Chip–disposable Hemocytometer (PeqLab). After 4 days of treatment with 5-aza-2'-deoxycytidine and MS-275 the cells were centrifuged (for 4 min at 1400 rpm), washed with PBS and the cell pellets were stored at -80°C.

For confirmation, experiments were independently repeated two times.

4.2 In vivo studies-Working with Mice

Tumor Xenografts studies

The animal studies were performed using *NOD.CB17-Prkdc^{scid}/NCrHsd* (NOD/SCID, Harlan Laboratories) mice in collaboration with the Pharmacology Department of the Medical University of Vienna. All animal experiments were carried out in accordance with protocols approved by Austrian law. The mice were kept in a controlled environment of light, humidity and temperature. Food and water were provided every day. Karpas-299 (ALK positive ALCL) human cells were grown as described above, centrifuged, washed and dissolved in sterile PBS to a concentration of 1×10^7 cells/ml. The cells were inoculated subcutaneously (100 μ l, 1×10^6 cells/injection) into the right and left flanks of the mice.

MS-275 was dissolved in 0.05 M HCL and 0.1% Tween and was given orally (p.o) in a concentration of 12 mg/kg/mouse.

5-Aza-2'-deoxycytidine - DNA methyltransferase inhibitor was suspended in PBS and was supplied intraperitoneal (i.p) in a concentration of 2.5 mg/kg/mouse.

Control mice were treated with 100 μ l of sterile PBS. Tumor range was measured when the tumors were palpable and was calculated by the formula: tumor width*tumor length. The weight of the mice was approximately 25 g/mouse and was measured at the beginning of the therapy.

Both therapies were adjusted regarding start and duration of the treatment in order to obtain optimal treatment procedures (Tables 1-3).

Xenograft 1	PBS control group of mice	MS-275 treated group of mice	Aza treated group of mice
Number of mice	2 mice	5 mice (treatment of one mouse was started 2 days after the treatment of the other four)*	5 mice (treatment of one mouse was started 2 days after the treatment of the other four)*
How many days after cell inoculation did the therapy start?	11 days	11 days	11 days
Tumor range at the beginning of the therapy	app. 1cm	app. 1cm	app. 1cm
Tumor range first measured after cell inoculation/for how long	11 days after inoculation/ measured 8 days/daily	11 days after inoculation/ measured 8 days/daily	11 days after inoculation/ measured 8 days/daily
How long was the treatment carried out?	8 days/daily treatment	8 days/daily treatment	8 days/daily treatment
How many mice died during the therapy?	no dead mice	no dead mice	two mice

Table 1

The therapy was started later because the tumors were ≤ 5 mm*
Mice were 10 weeks old at the beginning of the therapy

Xenograft 2	PBS control group of mice	MS-275 treated group of mice	Aza treated group of mice
Number of mice	5 PBS mice (3 mice 5-aza (i.p) ; 2 mice MS-275 (p.o))	6 mice	6 mice
How many days after cell inoculation did the therapy start?	3 days	3 days	3 days
Tumor range at the beginning of the therapy	no palpable tumors	no palpable tumors	no palpable tumors
Tumor range first measured after cell inoculation/for how long	MS-275 control group: 7days/6days; Aza control group:7days/3days;	7 days/6 days	7 days/3 days
How long was the treatment carried out?	MS-275 and Aza control groups: Mo-Fr; except weekends (MS-275: 10 times; Aza: 7 times of therapy)	Mo-Fr; no therapy during weekends (10 times of therapy)	Mo-Fr; no therapy during weekends (7 times of therapy)
How many mice died during the therapy?	no dead mice	one mouse	three mice

Table 2

Mice were 6 weeks old at the beginning of the therapy

Xenograft 3	PBS control group of mice	Aza early treated group of mice	Aza late treated group of mice
Number of mice	3 mice	3 mice	4 mice
How many days after cell inoculation did the therapy start?	6 days	4 days	6 days
Tumor range at the beginning of the therapy	no palpable tumors	no palpable tumors	palpable tumors
Tumor range first measured after cell inoculation/for how long	6 days/4 times (every 2nd day)	6 days/4times (every 2nd day)	6 days/ 4times (every second day)
How long was the treatment carried out?	4 times every second day; no therapy during weekends	5 times every second day; no therapy during weekends	4 times every second day; no therapy during weekends
How many mice died during the therapy ?	no dead mice	one mouse	one mouse

Table 3

Mice were 11 weeks old at the beginning of the therapy

4.3 Cell Cycle Analysis by Propidium Iodide (PI) Staining

About 10^5 to 10^6 cells were suspended in 500 μ l PI-buffer + RNAase (1:100) (Sigma) + PI (1:50) (ROTH) and incubated for 30 min at 37°C or 1 h at RT. 3 ml of PBS were added and cells were centrifuged at 1000 rpm for 5 min. The supernatant was removed. For flow analysis the cells were suspended in 500 ml PBS. The analysis was performed on a BD FACSCanto II flow cytometer using the BD FACS Diva Software.

Flow analysis:

Approximate settings (on FACS):

FL1: 570 V log. (E.g. to detect GFP)

FL2: 470 V linear

PI- buffer:

0,1% Na-citrat (Na-citrat dihydrate p.a. 113,9 mg/100 ml)

0,1% Triton X-100, for molecular biology (c=1,065 g/ml)

0,1% Rnase (Ribonuclease), (DNAase free)

Dilute in 1xPBS and filtrate through filter 0,2 μ m (sterile)

For 100 ml Puffer:

113,9 mg tri- Natriumcitrat dihydrate

100 mg Triton X-100

100 mg RNAse

Dissolve in 100 ml 1xPBS, sterile filtrate (50 ml Falcon), store at 4°C.

Propidiumiodide Stocksolution:

Dissolve 1 mg/ml in PBS, filter sterile and store at 4°C.

4.4 Work with DNA

4.4.1 Isolation of genomic DNA

This method was used for isolation of genomic DNA from cells and tissue. For isolation from tissues, the tissue was pulverized in liquid nitrogen. The pellet (from

cells or tissue) was dissolved in 500 µl genomic DNA isolation buffer. 20 µg/ml RNAase A was added to the mixture and incubated at 37°C for one hour. Then, 500 µg/ml Proteinase K was added and the DNA was incubated o.n at 55°C. Phenol/chloroform extraction was performed in phase lock tubes. 1x Vol of phenol was added to the DNA mixture and the solution was centrifuged for 10 min at 13.000 rpm at 4°C. The upper phase that contained the DNA was transferred to another tube and chloroform was added followed by centrifugation. The DNA was precipitated with 1x Vol isopropanol and centrifuged as described above. The DNA pellet was washed with 1 ml of 75% ethanol and centrifuged again. The supernatant was carefully removed and the DNA pellet was dried at room temperature. The DNA was dissolved in sterile water and incubated at 37°C until completely dissolved. The DNA concentration was measured with a Nanodrop 2000 (Peglab biotechnologies).

Genomic DNA Isolation Buffer:

0.4 M NaCl (5 M stock)

0.2% SDS (10% stock)

0.1 M Tris (1 M Tris pH 8.3)

5 mM EDTA (0.5 M EDTA)

4.4.2 Bisulfite Conversion and methylated DNA analysis by COBRA

For bisulfite conversion the EZ DNA Methylation™ Kit (Zymo Research) was used according to the manufacturer's protocol.

The following program was used for o.n incubation in a thermocycler: (95°C for 30 sec, 50°C for 60 min) x 16 cycles, then the temperature was reduced to 4°C.

For PCR, 2 µl of the bisulfite converted DNA were used. The PCR product was analyzed on a 1.5-2% agarose gel. Primers for amplification of bisulfite converted DNA were designed using the SEQUENOM EpiDesigner program (<http://www.epidesigner.com>).

For digestion, enzymes cutting the target sequence, containing CGs, were used (WNT2: HhaI (Fermentas); p16: TaqI (New England Bio Labs). As control fully methylated (SssI treated) and non-methylated (PBMC) DNA were used.

15 µl of the PCR product in TAE were used for the digestion following the protocol

bellow. To analyze the methylation status, 15 µl of the restriction were loaded onto a 10% Polyacrylamide Gel or onto an Agilent DNA chip (1000 series). 1 µl of the restriction product were used to obtain a virtual DNA gel (analysis program: 2100 expert). Chip preparation was carried out according to protocol.

PCR:

2.5 µl Buffer

2.5 µl MgCl₂

0.5 µl dNTPs

1 µl (Primer fo+re)

0.125 µl Ampli Taq Gold Polymerase (Applied Biosynthesis)

16.5 µl H₂O

Restriction enzyme digestion:

(15 µl PCR product)

2 µl Buffer

0.5 µl BSA

0.5 µl Res. Enzyme

2 µl H₂O

(or without BSA with 2.5 µl H₂O)

10% Polyacrylamide Gel (40 ml):

H₂O-15.6 ml

30% acrylamide mix-16 ml

5x TBE-8 ml

10% Ammonium persulfate (APS)-280 µl

TEMED-14 µl

4.4.3 Methylation-sensitive high resolution melting (MS-HRM)

Preparation of control samples

SssI treated DNA were used as methylation positive control and PBMCs as unmethylated negative control. A standard dilution series of 100% to 10%, 1% and 0,1% methylated DNA in a background of unmethylated DNA were prepared by serially diluting the methylated positive control into the unmethylated control.

Primer design for bisulfite PCR amplification

Primer preparation:

- Primer should have a length between 20- 40 nt
- Include a limited number of CG nucleotides (1-3 nt) into the primer sequence at the 5' end. If it is not possible to include only few CGs, mismatch the C from CG with T
- The correct melting temperature has to be checked out via qRT-PCR
- Programs for calculating the correct annealing temperature:
<http://www.promega.com/biomath/calc11.htm>
https://www.finnzymes.fi/tm_determination.html

Primer sequence used for the analysis:

p16HRMfo: GGAGTTTTTCGGTTGATTGGTTGGTT(25)

p16HRMre: AACACGCCCCGCACCTCCTCTA(22)

PCR reaction mixture:

(preparing a mastermix by adding the following reagents to 2 µl of DNA)

10 µl	KAPA SYBR FAST qPCR Mastermix (2x)
1 µl	Primer
7 µl	H ₂ O
20 µl	total

4.5 Work with Proteins

4.5.1 Western Blot/Protein Extraction

From tissue

Tumor tissue was put into a dounce homogenizer. 500 µl Hunt buffer and 5 µl protease (c=10 mg/ml) (Roche) and phosphatase inhibitor (Phosphatase Inhibitor Cocktail Tablets-Roche) were added. The tissue was dounced 10-15 times until well dissolved. The homogenate was frozen in liquid nitrogen and then put on ice to thaw.

From cells

The pellet was dissolved in 100-200 µl Hunt buffer with previously added protease and phosphatase inhibitor. The pellet was frozen in liquid nitrogen, put at 37°C to thaw, then frozen again and put on ice until completely thawed.

Then the solution (*from tissue or cells*) was centrifuged for 10 min at 4°C, the supernatant was taken out and the protein concentration was measured using Coomassie Plus Protein Assay Reagent (Pierce) diluted with H₂O (1:1) and the

protein concentration was measured with a spectrophotometer (HITACHI) or Nanodrop 2000 (Peglab biotechnologies). Samples were diluted with protein loading dye-2xSDS gel-loading dye (1:1), heated for 5 min at 95°C and loaded onto an already prepared SDS-PAGE (5-15%) gradient gel (50 µg of protein per lane).

2xSDS gel-loading dye, reduced:

100 mM Tris-HCl pH 6.8

200 mM DTT

4% SDS

20% Glycerin

Bromphenolblau (up to 20 ml)

Reduced gel loading dyes protonated and should be stored at -80°C.

Western Blot

The gel was transferred onto a nitrocellulose membrane (wet o.n transfer at 25V). The membrane was stained with Ponceau Red and washed with TBS-T. It was blocked with Blocking solution and incubated with primary antibody o.n. It was washed again with TBS-T (3x5 min) and incubated with secondary antibody for one hour. The membrane was incubated with chemoluminiscent solution ECL plus (GE Healthcare-1:40 diluted) and the signal was detected with the Lumi Analyst (Roche Applied Science).

Hunt Buffer:

20 mM Tris pH 8

100 mM NaCl

1 mM EDTA

0.5% NP-40 (stock 10%, fresh)

Protease inhibitor-Complete (Roche)

10X TBS (1l):

60.5 g Tris Base

90 g NaCl

With HCl to pH 7.5

TBS-T:

1x TBS

0.1% Tween

Blocking Solution:

10 g (5%) milk powder

400 µl Na-Azid (10%)

Fill up with TBS-T to 200 ml

4.5.2 Isolation of Core Histones

Tumor tissues were pulverized with liquid nitrogen. 1 ml Nuclear Isolation Buffer and 20 µl protease inhibitor (c=10 mg/ml) were added and the solution was centrifuged for 5 min, 500xg at 4°C. The supernatant was discarded and 100 µl of 0.4N H₂SO₄ were added to each tube. Afterwards, the tubes were put on ice for one hour. The solution was centrifuged for 5 min, 7500 rpm, at 4°C. The acid soluble supernatant was collected in 2 ml eppendorf tubes and precipitated with 10x Vol ice-cold acetone. The proteins were precipitated at -20°C o.n. Then they were centrifuged for 10 min, max speed, at 4°C. The supernatant was discarded and the pellets were washed with 500 µl acetone. Then, it was centrifuged again and the supernatant was discarded. The pellets were dried at room temperature and resuspended in 200 µl sterile water. The protein concentration was measured using the Nanodrop 2000 (Peglab biotechnologies). Histones were stored at -80°C until usage.

Nuclear Isolation Buffer:

10 mM Hepes pH 7.4

150 mM NaCl

1.5 mM MgCl₂

0.5% NP-40

4.6 Immunohistochemistry

The paraffin slides were put at 56°C o.n. For deparaffinization the slides were washed three times for 10 min in xylol, then rinsed in 100% (three times), 70%, and 50% EtOH and washed two times with distilled water. For antigen retrieval, the slides

were put into citrate buffer (pH 6) and heated in the autoclave. After cooling down at RT for 30-45 min, the slides were washed 3 times with PBS. To block endogenous peroxidase, the slides were washed with 3% H₂O₂ (Gatt-Koller) for 10 min and then 3 times with PBS. The staining area was marked with a PAP pen (Sigma-Aldrich). Then, the slides were blocked with Avidin Block (Zamponi Diagnostik) for 10 min and washed again 3 times with PBS. Biotin Block was added for 10 min followed by 3 times PBS washing. Then, the slides were blocked with Super Block for max. 7 min and washed 3 times. The slides were incubated with primary Antibody diluted in 1% PBS/BSA o.n at 4°C. Slides were washed 5 times and incubated with secondary antibody (anti-polyvalent biotinylated antibody) for 10 min. After washing 3 times the slides were incubated for 10 min with Streptavidin HRP (streptavidin peroxidase). Slides were stained with AEC (ID-Labs) and observed under the microscope to achieve optimal staining results and then stopped with distilled water. The slides were put in haemalaun solution (Merck) for 30 sec and washed with water. To complete the procedure the slides were mounted using aquatex (Merck) and cover glasses.

4.7 Antibodies

Antibodies used for Western blot

Antibody	Dilution WB	2nd antibody	Company
Dnmt1 goat polyclonal	1/1000	goat	Santa Cruz
HDAC1	1/1000	mouse	Seiser laboratory
HDAC2	1/1000	mouse	Seiser laboratory
HDAC3	1/1000	mouse	Seiser laboratory
β-Actin	1/1000	rabbit	Cell Signaling
NPM1	1/1000	mouse	obtained from Hematopathology 3J
NPM-ALK	1/1000	mouse	obtained from Hematopathology 3J
p-STAT3 (Tyr 705)	1/1000 and 1/5000	rabbit	Cell Signaling
p-Erk	1/1000	rabbit	Cell Signaling
Erk total	1/1000	rabbit	Cell Signaling
H4 global	1/1000	rabbit	Cell Signaling
Ac H3(K9)	1/1000	rabbit	Cell Signaling

Antibodies used for Immunohistochemistry

Antibody	Dilution WB	Company
Ki 67	1/1000	Cell Signaling
Caspase 3	1/200	Cell Signaling

H&E staining was performed by the Clinical Pathology-Immunohistochemistry department at the Vienna's General Hospital.

5. Results

We were interested in studying the effects of two different types of epigenetic drugs on ALCL *in vitro* and *in vivo*. Our research was centered on the following objectives:

- 1) To analyze the effect of the inhibitors on cell proliferation *in vitro*.
- 2) To perform an *in vivo* study using a Xenograft model.
- 3) To analyze the *in vivo* effects of the drugs (DNA methylation status of candidate genes, depletion of DNMT1 after Aza treatment, hyperacetylation of histones after MS-275 treatment).

5.1 *In vitro* and *in vivo* analysis after treatment with Aza

5.1.1 Effects of Aza on ALK positive ALCL and ALK negative ALCL cell lines

To evaluate the effects of Aza we performed *in vitro* studies with ALK positive ALCL and ALK negative ALCL cell lines. We have used two ALK positive (Karpas-299 and SR-786) and one ALK negative (Mac-2A) cell lines. Aza was administered once at the beginning of the treatment. In the Aza treated cells after 4 days of treatment the number of treated cells was reduced to around 55% less Mac-2A and SR-786 cells and 36% less Karpas-299 cells compared to PBS treated cells (Figure 3). In conclusion all treated cell lines were highly sensitive to Aza treatment.

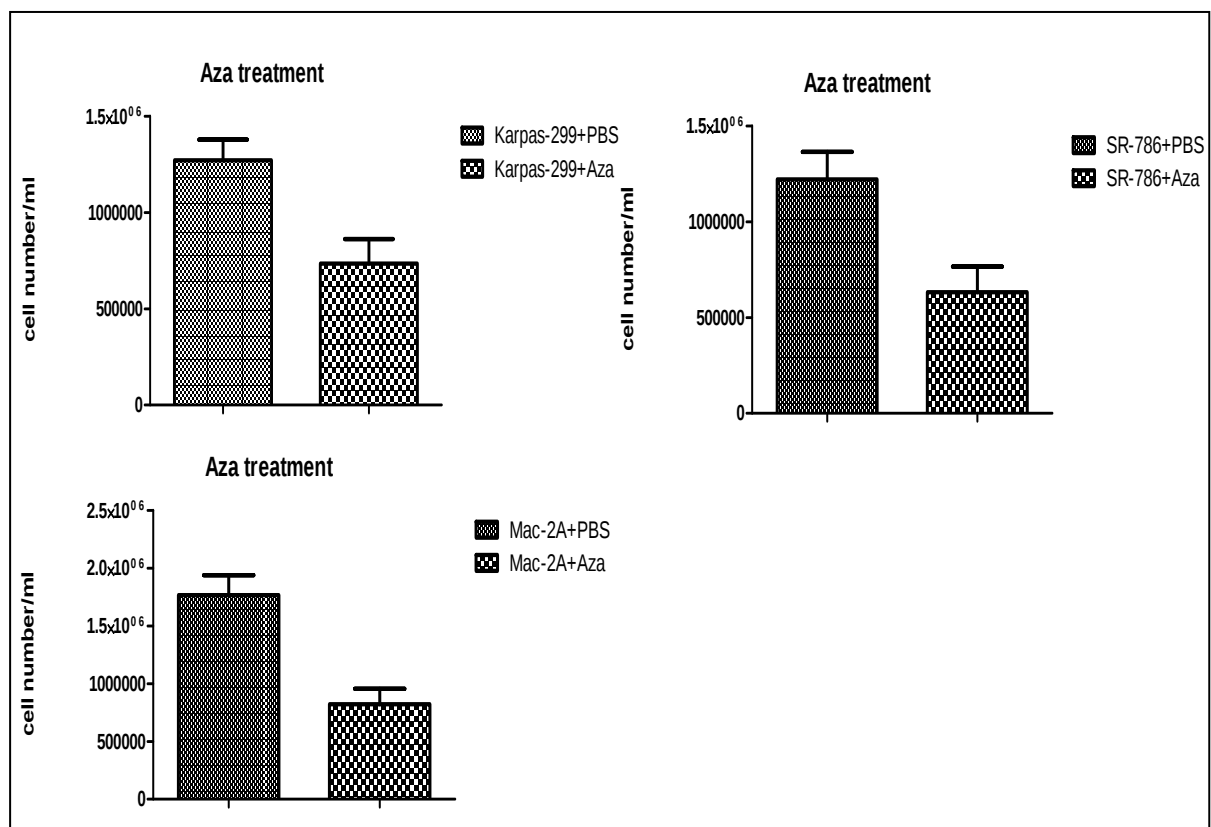


Figure 3 Treatment of ALK positive ALCL (Karpas-299 and SR-786) and ALK negative (Mac-2A) ALCL cell lines with Aza. Cells were treated for 4 days with 2 μ l of 1 mM Aza (c=1 μ M). Controls were treated with 2 μ l of sterile PBS.

5.1.2 Effects of Aza in a Xenograft model

Encouraged from our *in vitro* studies we performed *in vivo*-Xenografts using NOD/SCID-mice. ALK positive ALCL cells (Karpas-299) were inoculated into the mice. The mice were kept and treated as described in material and methods. In the first Xenograft the therapy was started 11 days after tumor cell inoculation, when palpable tumors were observed. Aza treatment did not achieve our expectations, because the tumors were aggressive and in advanced stage, when the treatment was started (more information about treatment and duration see Table 1). The mice involved in the next Xenograft were treated 3 days after tumor cell inoculation and did not have palpable tumors at the beginning of the therapy. This treatment schedule was very effective and was able to stop tumor growth in most of the treated mice compared to non-treated control mice, however with cytotoxic side effects (for more information about treatment and duration see Table 2). In order to obtain tumor

material for histological and molecular analyses we performed another Xenograft model with a different treatment schedule. The first group Aza early was treated 4 days after tumor cell inoculation and had no palpable tumors at the beginning of the treatment. The second group Aza late had palpable tumors at the beginning of the treatment and was treated 6 days after inoculation. The last group was the PBS control group where the PBS implementation started at the same time with the Aza late group. The Aza early was treated 5 times every second day (no treatment during weekends), whereas the Aza late and PBS control group was treated one time less, 4 times, every second day (no treatment during weekends) (for more information about treatment and duration see Table 3). This Xenograft showed reduced tumor growth and weight in treated compared to untreated mice, less cytotoxicity and we could obtain valuable tumor material for our following investigations. The figures below refer to the last Xenograft model (see also supplementary tables S1-S6 and supplementary figures S1 and S5 for more details about Aza-Xenograft treatments). The tumors of Aza treated mice were smaller in size and weight compared with the non-treated controls (Figure 4).

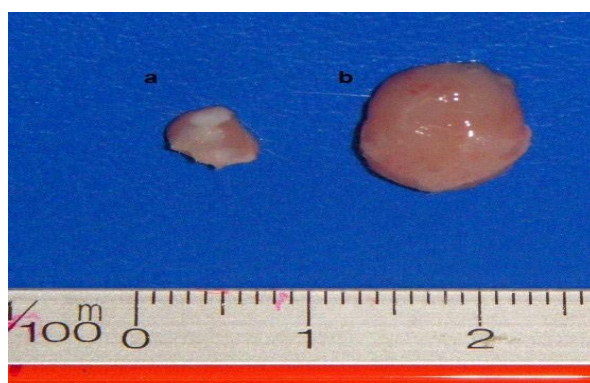


Figure 4 Tumor photograph from mice. (a) Aza early treated mouse (b) PBS-control mouse

Aza treated tumors showed significant size and weight reduction compared to the non-treated controls (Figure 5,6).

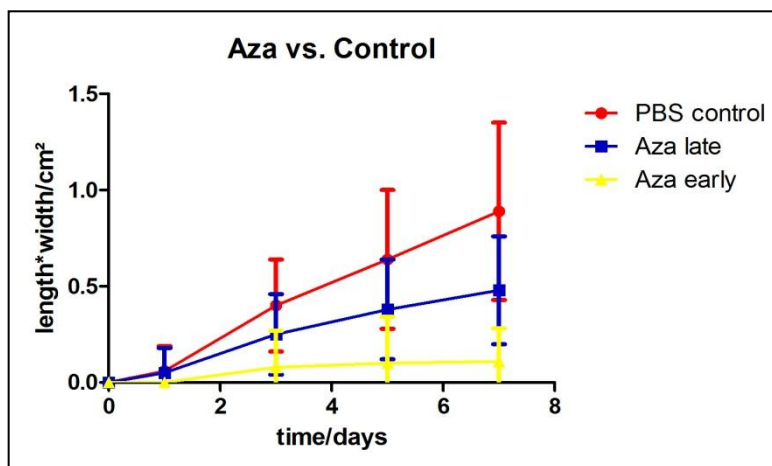


Figure 5 Treatment of mice with Aza. The tumor range (length*weight) was calculated as average of all tumor ranges. The tumor range was first measured 6 days after cell inoculation. (see also Table.3-Material and Methods for additional information).

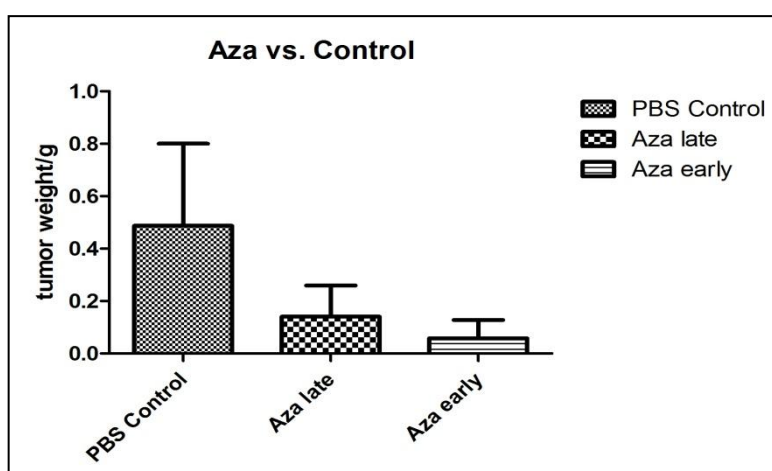


Figure 6 Tumor weight of Aza treated vs. non-treated mice. After Aza therapy mice were sacrificed and the tumors (n(Aza early)=2; n(Aza late)=5; n(PBS control)=5) were isolated and measured. The figure shows tumor weight average of all three groups of mice. Tumor weight is reduced in the treated compared with the non-treated group.

To conclude, tumor growth and tumor weight reduction was observed in the both treated groups, whereas the non-treated group showed increased tumor growth and tumor weight.

5.1.3 FACS analysis of Aza treated mice

To analyze the effect of Aza on proliferation we isolated cells from the tumor tissue and performed FACS analysis. First, our results demonstrated that there are more cells in the G1 phase in treated compared with the non-treated mice (Figure 7a). Untreated tumors proliferate to a highly extend and more tumor cells are present in the S-phase from the non-treated then the treated mice (Figure 7b). Sub G1 phase showed an increased number of apoptotic cells present in the treated compared with

the non-treated tumors from control mice (Figure 7c) (see also supplementary figure S2 for FACS analyses from the first Xenograft treatment).

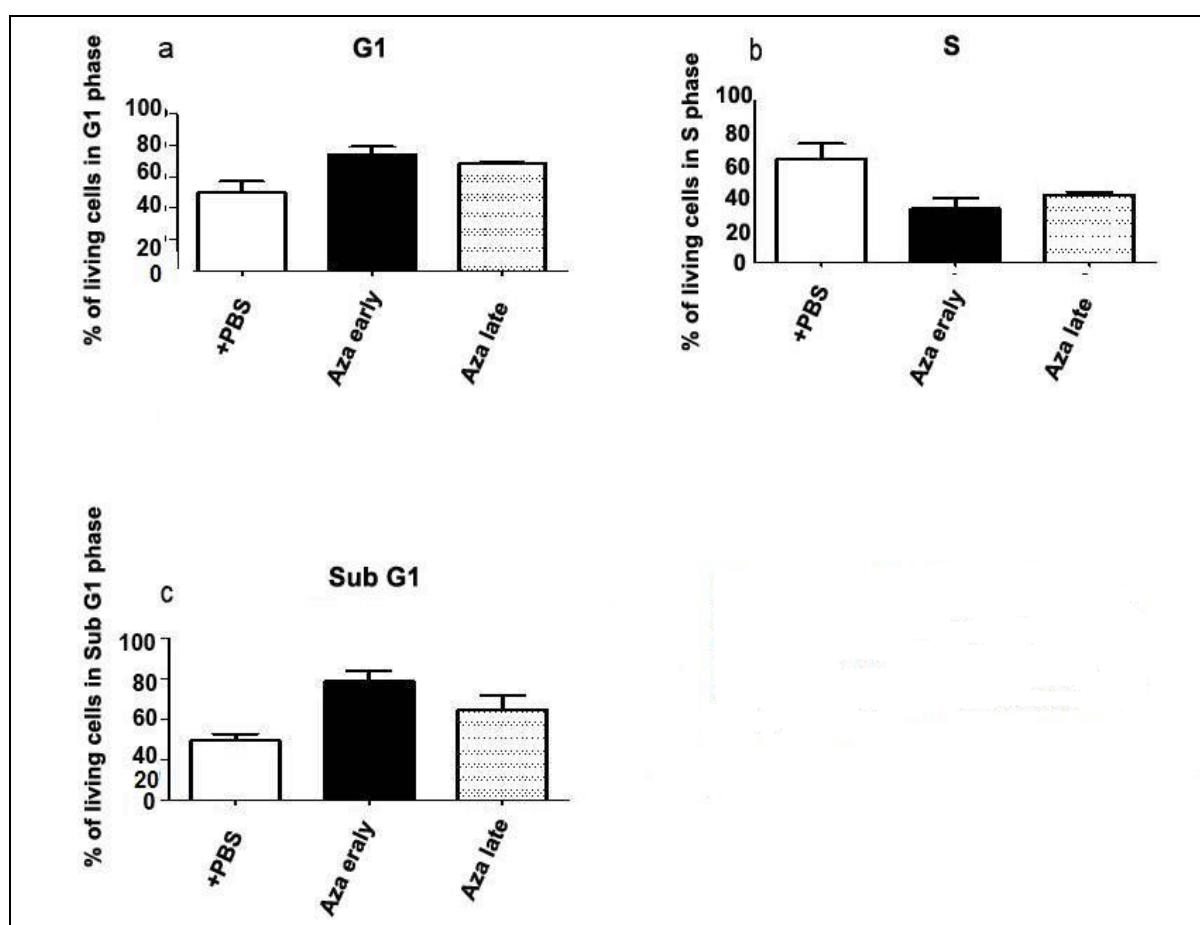


Figure 7 FACS analyses of Xenograft tumors (a) An increased number of cells is observed in the G1 Phase. (b) The S-phase shows increased number of dividing cells in tumors (PBS) compared with Aza treated mice. (c) Sub G1 shows more nuclei of apoptotic cells in treated than in untreated mice. Aza early group of mice was treated two days earlier than the Aza late group (see Table 3).

Therefore, our data show that Aza treatment reduces the proliferation of tumor cells and causes increased apoptosis.

5.1.4 DNA methylation status of tumor suppressor genes

The cancer genome has an altered epigenetic landscape. Several tumor suppressor genes such as p16 or, RASSF1A show changes in epigenetic patterns and sustain epigenetic silencing (87-89). We performed DNA methylation analysis using bisulfite converted DNA isolated from the Xenografts of untreated and Aza treated mice to analyze the methylation status of candidate tumor suppressor genes usually silenced in ALCL. It has been shown that WNT2 is hypermethylated in ALCL cell lines

(M.Hassler, personal communication). We confirmed, that WNT2 is less methylated in mice after treatment with Aza compared to the non-treated control group (Fig. 8).

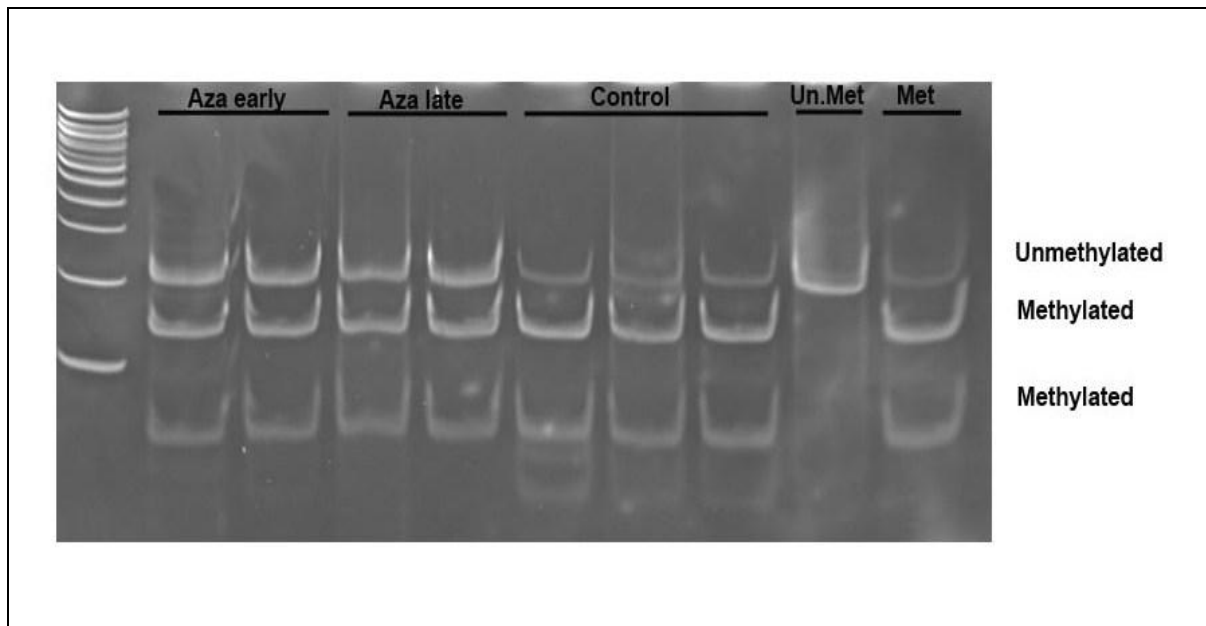


Figure 8 COBRA analysis of WNT2. Analysis of the methylation status of bisulfite converted DNA of the WNT2 promoter. The non-treated group (control) showed higher methylation levels compared with the treated groups (early and late treated) as seen by a higher intensity of the undigested band representing unmethylated DNA. Unmethylated-PBMCs (un.met) and methylated-SSSI treated DNA (met) were used to indicate non-methylated and completely methylated samples.

We have also confirmed the methylation status of WNT2 using the Agilent DNA-Chip 1000, which is a more sensitive method for detection of the methylation status of candidate genes. In the figure below the reduction of the methylation status in the treated mice, but not in the controls, was confirmed (Figure 9a).

In the electropherogram we have compared the peaks of the unmethylated DNA with the controls and the treated mice. It was observed that the peak seen at 80 seconds, which represents the unmethylated PCR product, is also present in the Aza treated mice, whereas in controls this peak is very low. This also confirms that the methylation status of WNT2 is lower after Aza treatment (Figure 9b).

a.



b.

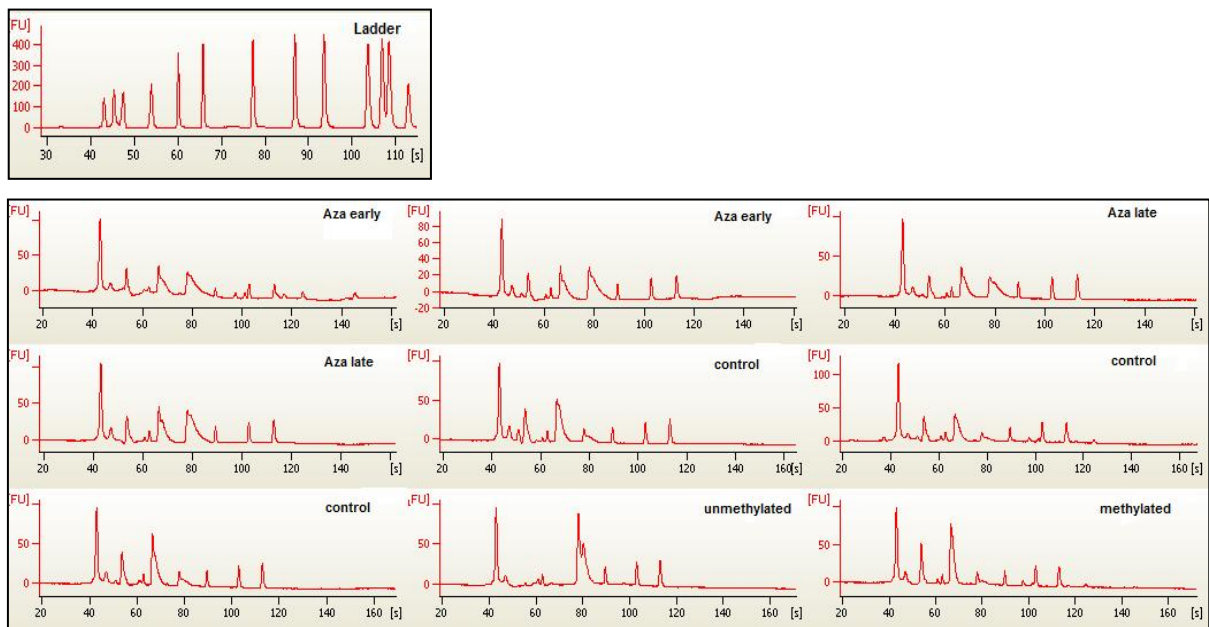


Figure 9 DNA methylation analyses. (a) DNA methylation analysis of WNT2 using DNA Chip-1000. The methylation status of the treated (aza early, aza late) was decreased as compared to non-treated (control) groups of mice. As controls methylated (SSSI treated) and unmethylated (bisulfite treated-PBMCs) DNA were used. (b) Electropherogram of WNT2 using DNA Chip analysis. The peak seen at 80 seconds, which represents the unmethylated product, is higher in treated mice, whereas in the methylated control sample there is a higher peak between 60 and 80 seconds, representing the non-methylated product, which is also observed in the non-treated controls.

Furthermore, the methylation status of p16, a tumor suppressor gene, which is found to be hypermethylated in diverse cancers such as multiple myeloma, T-cell lymphomas and colorectal cancer and which is also associated with lymphocytic and plasmacytic malignancies (70,88-90), was analyzed using methylation-sensitive high

resolution melting (MS-HRM). Standards with different methylation levels were used. 100% methylated (SssI treated bisulfite DNA) was diluted to 10%, 1% 0,1% standards (see Materials and Methods). T-cells (bisulfite treated PBMCs) were used as unmethylated control. We have used DNA samples from the two Aza treated groups Aza early and Aza late, and from the untreated PBS control group of mice. Untreated control mice (82L, 76L, 51L) have higher p16 methylation patterns compared with the treated group. Reduction in methylation patterns was also observed in the Aza late group (77L, 77R, 81R) compared with non-treated control group, but the methylation level was increased compared with the Aza early treated group (79L, 78L), which had the lowest methylation patterns in these three groups.

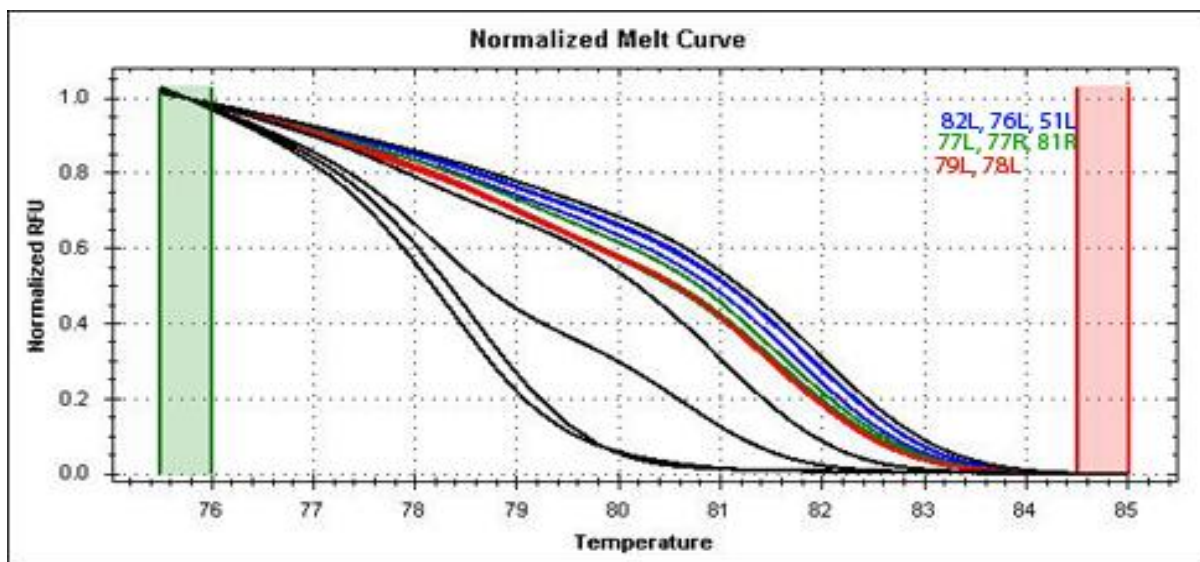


Figure 10 Melting curve analysis of p16. The figure shows differences in the methylation status between the two treated groups mouse Aza early: (79L, 78L) and Aza late: (77L, 77R, 81R) and non-treated: (82L, 76L, 51L) mice. Standards (black curves) diluted from 100% to 10%, 1% and 0,1% and also unmethylated (0%) were used to compare and confirm the methylation patterns of p16. Methylation patterns are increased in non-treated compared with Aza late and Aza early, where less methylation is observed.

In conclusion, DNA methyltransferase inhibitors can decrease hypermethylation levels of epigenetically regulated target genes *in vivo* in an ALCL Xenograft mouse model.

5.1.5 Protein analysis of Aza treated Xenografts

DNMT1 is a maintenance methyltransferase involved in copying the methylation pattern from the mother to the daughter cell. DNMT1 is covalently bound to DNA after Aza treatment and therefore depleted from the protein extract. Furthermore, it was suggested that DNMT1 might undergo proteosomal degradation after treatment with

Aza (91). Our analyses have also shown that there was no DNMT1 protein present after Aza treatment compared with the control group where DNMT1 protein was highly abundant in the protein extracts (Figure 11).



Figure 11 DNMT1 expression in mice treated with Aza. After treatment with Aza there was no Dnmt1 detectable in treated mice (Aza early, Aza late) compared with untreated control mice (Control). β -Actin is used as loading control.

These data indicate that Aza efficiently depleted DNMT1 *in vivo* in the tumor cells of the Xenograft (DNMT1 depletion was also observed after the first Xenograft treatment see supplementary figure S3).

5.1.6 Immunohistochemistry staining of Aza treated tumor tissues

In order to observe histological changes in Aza treated Xenograft tumors we performed immunohistochemistry staining. Observations regarding histology were treated mice made after staining with hematoxylin and eosin. We have observed more apoptotic areas in mice treated with Aza compared with untreated controls (Figure 12). Caspase 3 staining was performed to identify apoptotic areas in the tumors. As expected from H&E staining, Caspase 3 was highly expressed in treated mice (Figure 12). Ki-67, which is expressed in dividing cells, was used as a cellular marker for proliferation. The cells in untreated mice had a higher proliferation rate than the cells in treated mice (Figure 12) (supplementary figures S4 and S6 for more information about the Immunohistochemistry staining after the first two Aza treatments).

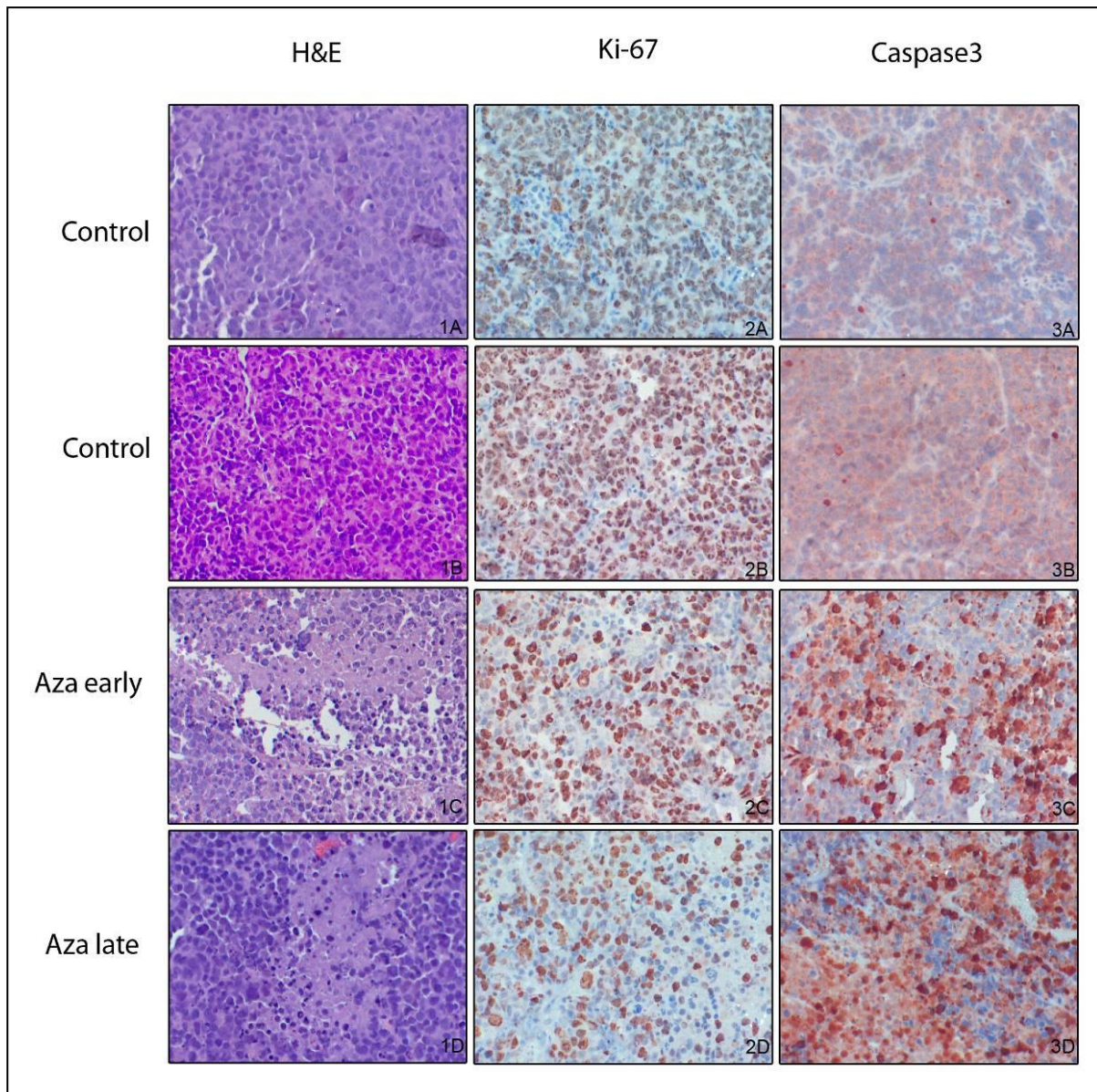


Figure 12 Immunohistochemistry staining of paraffin embedded tumor tissue sections in mice after treatment with Aza (early or late treatment). (C; D) are sections of two treated mice (early and late treated) compared with (A; B) sections of two non-treated mice. H&E staining showed increased apoptosis rates in treated (1C, 1D) compared with non-treated (1A, 1B). Ki-67 staining showed higher proliferation in non treated (2A, 2B) vs. treated (2C, 2D). Caspase 3 was higher expressed in treated mice (3C, 3D) compared with non treated mice (3A, 3B).

To conclude, it was observed that Aza treated mice showed increased apoptosis and Caspase 3 activity and decreased proliferation rates.

5.2 *In vitro* and *in vivo* analysis after treatment with MS-275

5.2.1 Effects of MS-275 on ALK positive ALCL and ALK negative ALCL cell lines

To evaluate the effects of MS-275 we also performed *in vitro* studies with ALK positive ALCL and ALK negative ALCL cell lines. The same tumor cell lines as described above (see Aza treatment) were used and MS-275 was administrated daily.

Reduced cell number was observed in MS-275 treated cells after 3 days of treatment; around 35% reduction in Karpas-299 and Mac-2A, whereas SR-786 cell number was reduced to 57% (Figure 13). Thus, ALCL cell lines were not just highly sensitive to Aza, but also to MS-275 treatment.

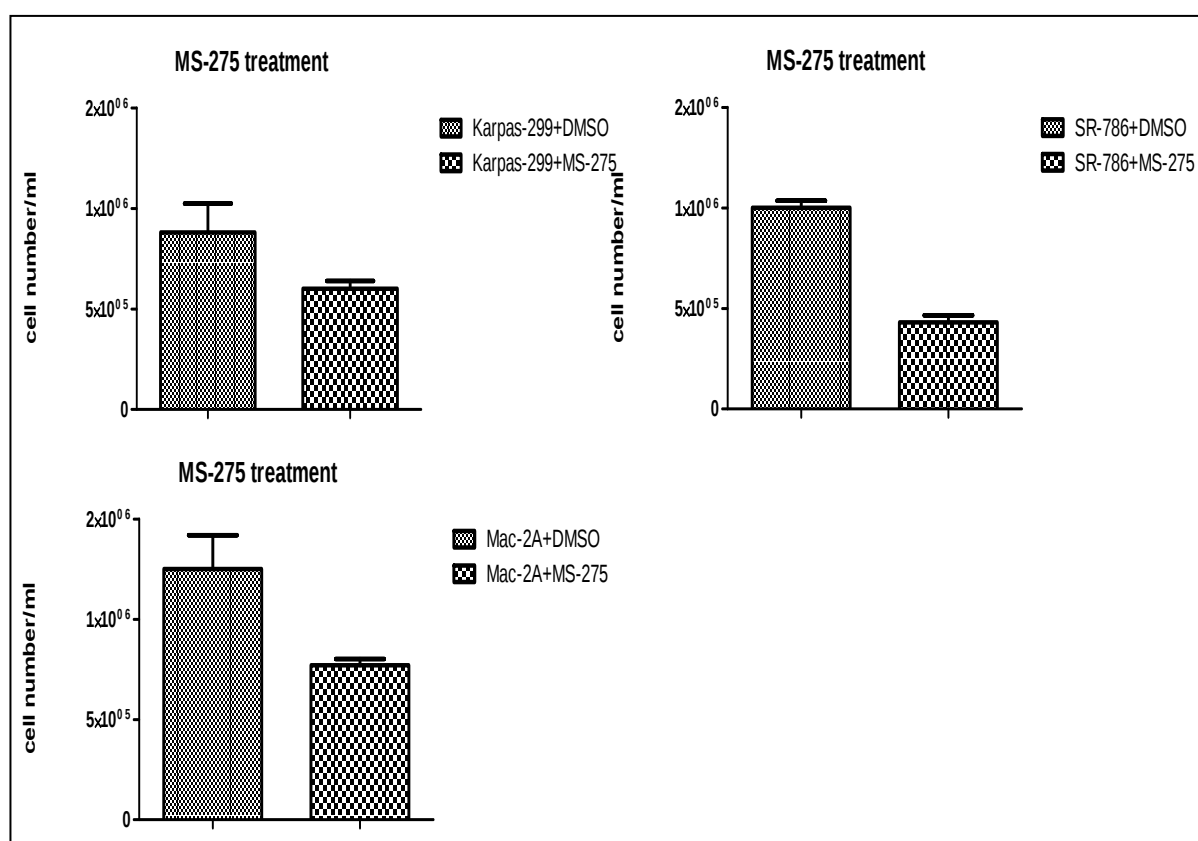


Figure 13 ALK positive (Karpas-299 and SR-786) and ALK negative (Mac-2A) cell lines with MS-275. Cells were treated for 3 days with 1 μ l of 1 mM MS-275 ($c=0.5 \mu$ M). Controls were treated with 1 μ l of DMSO.

5.2.2 In vitro protein analysis of MS-275 treated cell lines

We tested the NPM-ALK protein levels *in vitro* in cells treated with MS-275. ALK positive ALCL (Karpas-299 and SR-786) and ALK negative ALCL (Mac-2A) protein extracts were used. We observed decreased levels of NPM-ALK in the two ALK positive cell lines after 3 days of daily treatment with MS-275. The expression levels of NPM1 were slightly reduced after treatment in all three cell lines. There was no NPM1 protein detectable in PBMCs (Figure 14).

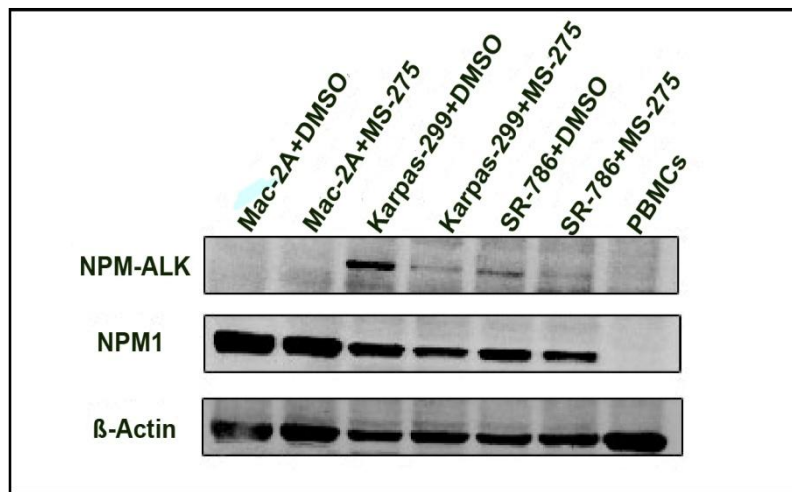


Figure 14 In vitro analyses. Protein expression profiles of NPM-ALK and NPM1 after 3 days of treatment with MS-275. NPM1 is expressed in treated and untreated (DMSO) protein extracts, whereas NPM-ALK is less expressed in treated ALK positive ALCL cell lines (Karpas-299 and SR-786).

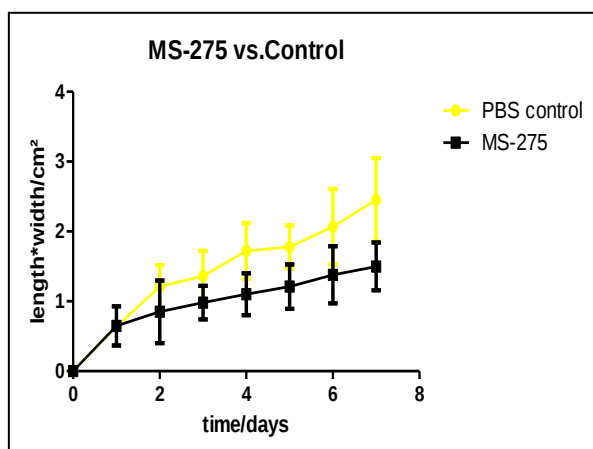
Our data suggest that treatment with MS-275 leads to a reduction in NPM-ALK protein levels. It will be interesting to analyze in the future whether this is a transcriptional effect or due to increased proteosomal degradation.

5.2.3 In vivo analysis of Xenograft mice treated with MS-275

To analyze the effectiveness of MS-275 treatment *in vivo* we also performed Xenografts in mice. In the first Xenograft the MS-275 therapy started 11 days after tumor cell inoculation at the time the tumors were palpable and aggressive. In the second Xenograft model the treatment started 3 days after tumor cell inoculation, where no palpable tumors at the beginning of the treatment were observed. Even though the two Xenografts showed slightly reduced tumor growth in treated mice, there was no visible difference in tumor weight between the treated and non-treated group of mice (Figure 15). The following figures refer to the first Xenograft treatment

(see also Table 1) and are comparable to the second set of Xenografts (data not shown).

a.



b.

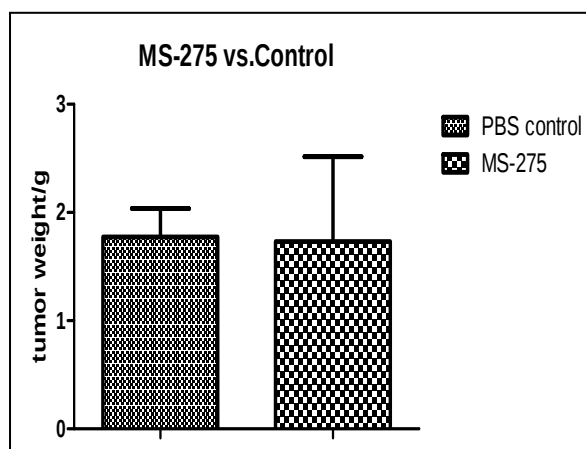


Figure 15 Tumor growth and weight in MS-275 treated compared with untreated mice. (a) Tumor growth reduction is observed in treated mice. (b) There is no difference in tumor weight in both groups: n(treated)=6, n(control)=4.

5.2.4 FACS analysis of MS-275 treated mice

Next we performed FACS analysis to analyze the cell cycle profile after MS-275 treatment. We observed a slight increase in G1 phase and a decrease in S-phase in MS-275 treated tumor cells compared to non-treated cells (Figure 15). Interestingly, we detected an increase in Sub G1 cells in control tumors, which might indicate an increased apoptosis due to the large size of the tumors. Thus, our data suggest that MS-275 treatment showed a slight effect on proliferation of Xenograft tumors. The effects observed were not significant and might be due to instability or bad uptake of the drug *in vivo*.

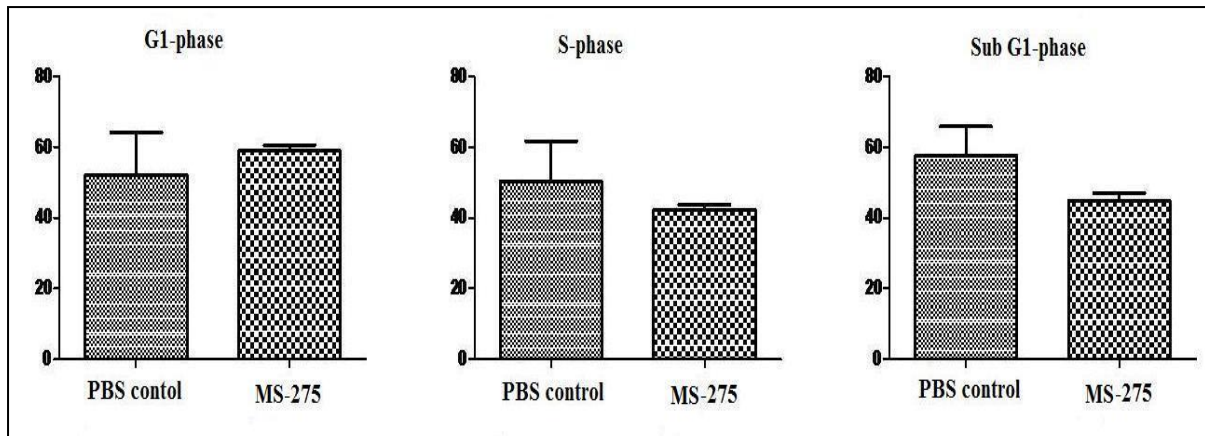


Figure 16 FACS analysis of MS-275 treated Xenografts. In G1-phase there is an increased number of cells of MS-275 treated mice compared with non-treated (PBS) control. In S-phase there are more dividing cells in tumors from untreated mice. Sub G1-phase shows fewer apoptotic cells in treated then in the control group.

5.2.5 Protein analysis of MS-275 treated Xenografts

Protein analyses were performed to analyze the acetylation patterns after the treatment with MS-275. We detected increased acetylation of hostone H4 in treated mice. As a loading control we used an antibody against global histone H3 (Figure 17). In summary our data show effects of the drug on global histone acetylation, although no significant changes in tumor growth or apoptosis could be detected.

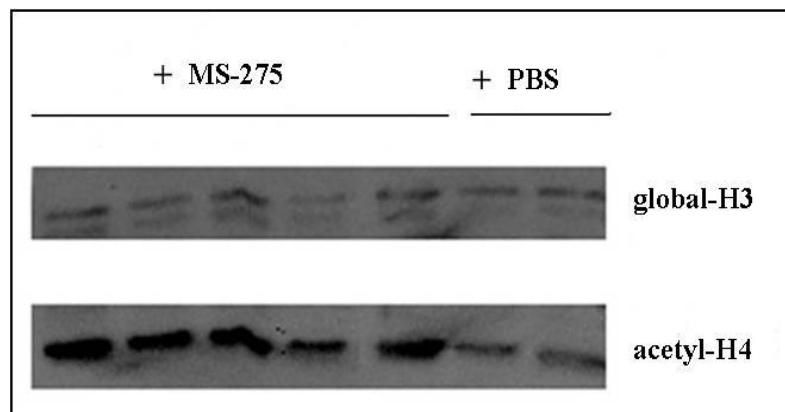


Figure 17 Analysis of histone acetylation levels in MS-275 treated mice. Increased acetylation can be observed in the treated mice compared with the non-treated PBS control group. The global H3 was used as loading control.

5.2.6 Immunohistochemistry staining of MS-275 treated mice tissues

To monitor the effects of MS-275 therapy we have also performed immunohistological staining using the same markers as above. Proliferation was slightly decreased in the treated mice (2C, 2D) as analyzed by Ki-67 staining. Apoptotic areas were detectable in both untreated and treated tumors as analyzed by Caspase 3 staining (3A-D) and most likely not an effect of the drug treatment but rather increased tumor size and bad nutrient supply of the tumor cells. We conclude that further experiments with different doses and schedules of MS-275 treatment will be necessary to demonstrate the efficacy of the drug *in vivo*.

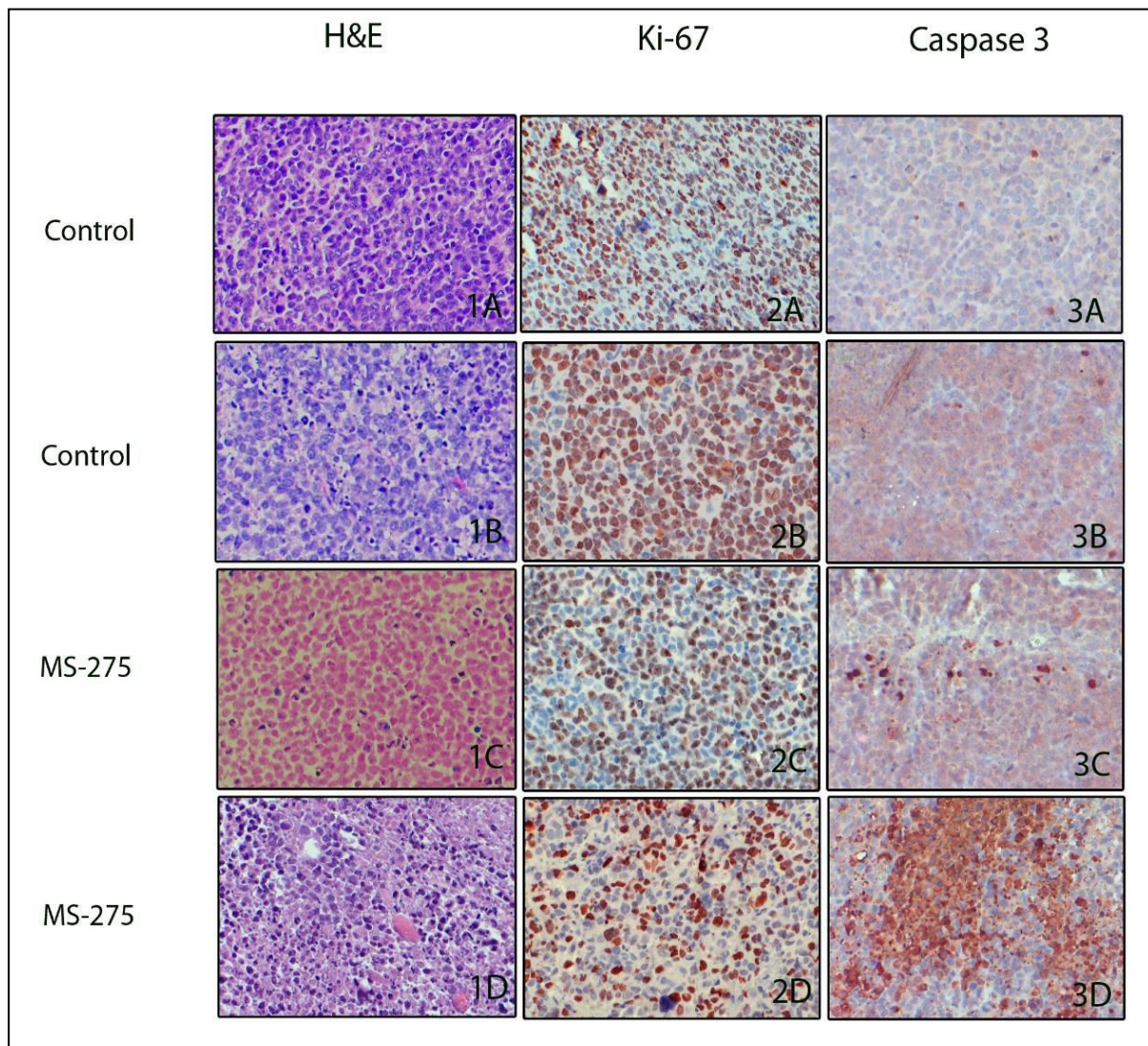


Figure 18 Immunohistochemistry Staining from MS-275 treated tumor tissues. Apoptosis areas observed in treated and non-treated mice (1A-D). There is a slightly lower proliferation rate in treated (2C, 2D), then in untreated mice (2A, 2B). Caspase 3 appeared higher in treated (3C, 3D), compared with untreated mice (3A, 3B).

6. Discussion

In our study, we aimed to analyze the effects of DNMT and HDAC inhibitors on ALCL cells *in vitro* and *in vivo*.

Hypermethylation of CpG islands in the cancer genome was described in the early nineteen's in a retinoblastoma model (92,93). It has been shown that decreasing of 5-methylcytosine in hypermethylated CpG islands in cancer is associated with lowering the number of the tumors (94).

Nowadays, epigenetic therapy is tested for treating diseases with epigenetic etiology such as different cancers. This therapy aims at reactivating silenced tumor suppressor genes. Aza showed promising results in various clinical trials (95-97). Clinical trials for the treatment of melanoma approved the effectiveness of Aza as demethylation agent, although the authors recommend prolonged low dose exposure in order to prevent cytotoxicity (98,99). Aza is also approved from the FDA for treating myelodysplastic syndromes (MDSs) in patients (97).

Encouraged from our *in vitro* studies with three different ALCL cell lines we implemented a Xenograft model using the ALK positive Karpas-299 cell line. We confirmed that tumor size and weight are decreased after treatment with Aza. We studied the methylation pattern of epigenetically silenced tumor suppressor genes. In line with previous studies that have shown demethylation of DNA after using this drug (100), we have also observed, a decrease in methylation patterns after Aza treatment in hypermethylated tumor suppressor genes, such as p16 and WNT2.

The tumor suppressor gene p16, located at chromosome 9p21, regulates the cell cycle with a functional unit constituting of p16, cyclin D1 and pRb. Early studies showed that p16 methylation in diverse myeloma decreases after treatment with Aza (90). Methylation of p16 has been detected in several lymphocytic and plasmacytic malignancies such as lymphoma, acute lymphocytic leukemia and multiple myeloma and shows a relation to the pathogenesis of these diseases. Demethylating agents such as Aza that revert p16 methylation are promising drugs for future therapy for treating such diseases (88).

Recent studies showed that NPM-ALK is involved in inducing DNA damage and irreversibly arresting the cell cycle (101). In the tumor development this process is called oncogene induced senescence and is related with activation of the ARF/p53

pathway, which is widely inhibited in lymphomas and leukemias. NPM–ALK lymphomagenesis showed p16 dependent accumulation of senescent cells in pre-malignant lesions and also lowering of the tumor latency in absence of p16 could be observed (101). However, we know that p16 is silenced in ALCL and its reactivation with upregulating either the histone demethylase-Jmjd3 or using demethylating agents could be a new strategy to reset the senescence program in tumors (101).

In our study we observed decreased methylation of the WNT2 promoter after treatment with Aza. From the literature it is known that WNT2 regulates diverse pathways and aberrant expression is associated with different types of cancer. In parathyroid carcinoma there is a defect in the WNT2/ β -catenin pathway caused by DNA methylation in APC and *in vitro* treatment with Aza induced APC expression, reduced active nonphosphorylated β -catenin, inhibited cell growth, and apoptosis (102).

Genexpression is regulated through the action of DNA methyltransferases. It was reported that DNMT1 expression levels are induced and regulated upon entry into the S-phase of the cell cycle, and its over expression is probably not responsible for the aberrant methylation in cancer cells (103). In our study we found depletion of the DNMT1 in Aza treated mice. Other studies reported that DNMT1 is overexpressed in gliomas (104), whereas DNMT3b, but not DNMT1 is over expressed in prostate cancer (105). Treatment with DNA methyltransferase inhibitors reduced the enzyme levels and caused re-expression of silenced tumor suppressor genes (104,105).

On the other hand, NPM–ALK is the most prominent fusion protein in ALCL and it downregulates T-cell receptor signaling molecules by STAT3 mediated gene transcription or epigenetic silencing, which is reverted by treating ALCL with Aza (106). It has been observed that in NPM–ALK positive cell lines STAT5A expression is inhibited directly by NPM-ALK induced methylation via STAT3. The same study also showed that treatment with Aza reactivates the gene (107). Another study suggested that NPM-ALK downregulates T-cell receptor signaling molecules by STAT3 mediated gene transcription or epigenetic silencing, which is reverted by treating ALCL with Aza (106). SHIP is also epigenetically regulated and silenced in ALCL and treatment with Aza in Karpas-299 cell line reactivates its expression in over 5 days (62).

MS-275 has been investigated in patients with acute leukemias, mostly AML. In

treated patients increased levels of p21 and Caspase 3 activity were observed (108). MS-275 is currently in the clinical Phase I for treatment of advanced leukemia and MDS (85). Clinical studies observed the effects of HDAC inhibitors on T-cell lymphoma, Hodgkin lymphoma, and myeloid malignancies and conclude that these drugs induce hyperacetylation and can be well tolerated from patients (109). Our results did also confirm slightly elevated acetylation upon usage of MS-275 in mouse xenograft tumors. In addition, our immunohistochemistry tissue staining indicated slightly higher Caspase 3 activity in treated and slightly increased proliferation in non-treated mice. In general, MS-275 treatment did only show limited inhibition of tumor growth and no weight reduction of tumors in most of the treated mice. The reasons for this could be not optimized dose of MS-275 or bad uptake of the drug. *In vitro* we showed that the cell number is reduced in all MS-275 treated cell lines and reduction of the NPM-ALK expression levels after treatment with MS-275 in the ALK positive cell lines. Further studies have to be done in order to improve the effectiveness of this drug in ALCL in dose/survival depended manner and also investigation on metabolic pathway and uptake of MS-275.

Many studies reviewed the effects of epigenetic drugs and combination therapy (HDAC and DNMT inhibitors) and their efficacy for treatment of cancer (110,111). Future approaches in this field might include both genetic therapy, which includes: small-molecule inhibitors of oncogenic signals, antibodies to surface components and intercellular communicating factors, and molecularly defined vaccines (112) and epigenetic therapy (113) for successful treatment of cancer.

In conclusion, epigenetic therapy showed promising results for the treatment of ALCL in mice and the future perspectives in this field should focus on adapting epigenetic therapy that can be effectively used and well tolerated by cancer patients.

7. Supplementary

7.1 Aza treatment

7.1.1 Xenograft 1

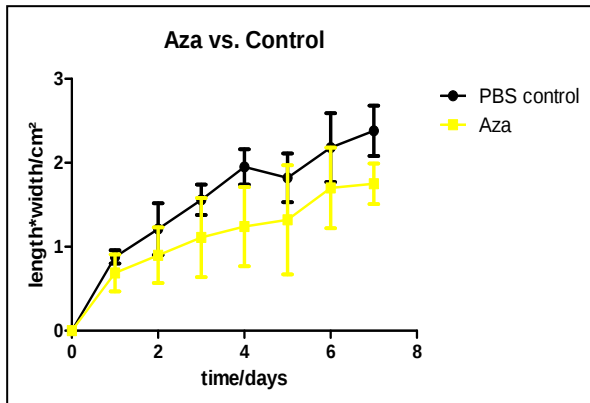
Aza												
No				Day 1						Day 3		
	Tu Length	Width	Circ.	Tu Length	Width	Circ.	Tu Length	Width	Circ.	Tu Length	Width	Circ.
140 R	0,95	1,3	1,24	1	1,4	1,40	1,2	1,75	2,10	1,25	1,8	2,25
140 L	1,1	0,9	0,99	1,3	1,1	1,43	1,5	1,3	1,95	1,35	1,2	1,62
143 R	0,95	0,75	0,71	0,9	0,9	0,81	1,05	0,9	0,95	1	1	1,00
143 L	0,65	0,9	0,59	1,1	0,85	0,94	1,1	1,05	1,16	1,1	1,2	1,32
144/145 R	0,75	0,7	0,53	1,1	0,9	0,99	1,3	0,9	1,17	1,2	1	1,20
144/145 L	0,8	0,7	0,56	0,9	0,8	0,72	1	1	1,00	1,15	1,1	1,27
147 R	1,05	0,85	0,89	1,2	1,1	1,32						
147 L	0,7	1	0,70	0,85	0,75	0,64						
156 R	0,6	0,5	0,30	0,6	0,6	0,36	0,6	0,6	0,36	0,7	0,55	0,39
156 L	1	0,9	0,90	0,95	0,95	0,90	1,5	0,8	1,20	1,5	1,25	1,88
MITTELWERT			0,74			0,95			1,24			1,36
148 R	1,1	0,75	0,83	1,1	0,9	0,99	1,3	1,1	1,43	1,4	1,5	2,10
148 L	0,85	1,1	0,94	1,3	1,1	1,43	1,4	1,2	1,68	1,5	1,2	1,80
MITTELWERT			0,88			1,21			1,56			1,95
No	Day 4			Day 5			Day 6			Day 7		
	Tu Length	Width	Circ.	Tu Length	Width	Circ.	Tu Length	Width	Circ.	Tu Length	Width	Circ.
140 R	1,35	1,75	2,36	1,2	1,55	1,86	1,3	1,65	2,15	1,2	1,15	1,38
140 L	1,5	1,4	2,10	1,6	1,3	2,08	1,55	1,3	2,02	1,3	1,3	1,69
143 R	1	0,95	0,95									
143 L	1	1	1,00									
144/145 R	1,25	1,1	1,38	1,4	1,4	1,96	1,4	1,2	1,68	1,2	1,1	1,32
144/145 L	1,25	1,05	1,31	1,2	1,05	1,26	1,3	1,2	1,56	1,15	1,15	1,32
147 R												
147 L												
156 R	0,65	0,55	0,36	1,05	1,05	1,10						
156 L	1,55	1,4	2,17	1,45	1,45	2,10						
MITTELWERT			1,45			1,73			1,85			1,43
148 R	1,4	1,15	1,61	1,4	1,35	1,89	1,55	1,4	2,17	1,6	1,3	2,08
148 L	1,5	1,35	2,03	1,7	1,45	2,47	1,85	1,4	2,59	1,55	1,2	1,86
MITTELWERT			1,82			2,18			2,38			1,97

Table S1 Tumor growth measurement during Aza treatment.

Tu weight	g
140 R	
140 L	1,91
144/145 R	1,17
144/145 L	0,77
156 R	
156 L	
MITTELWERT	1,28
148 R	1,85
148 L	1,95
149 R	1,91
149 L	1,38
MITTELWERT	1,77

Table S2 Tumor weight measurement after Aza treatment.

a.



b.

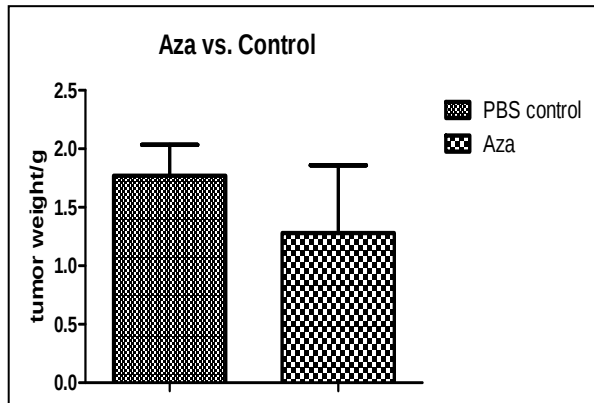


Figure S1 (a) Treatment of mice with Aza. Decreased tumor growth was observed in treated mice compared with the untreated (PBS) control group. (b) Tumor weight measurements in treated (n=3) vs. untreated mice (n=4). Tumor weight and growth reduction was observed in the Aza treated mice.

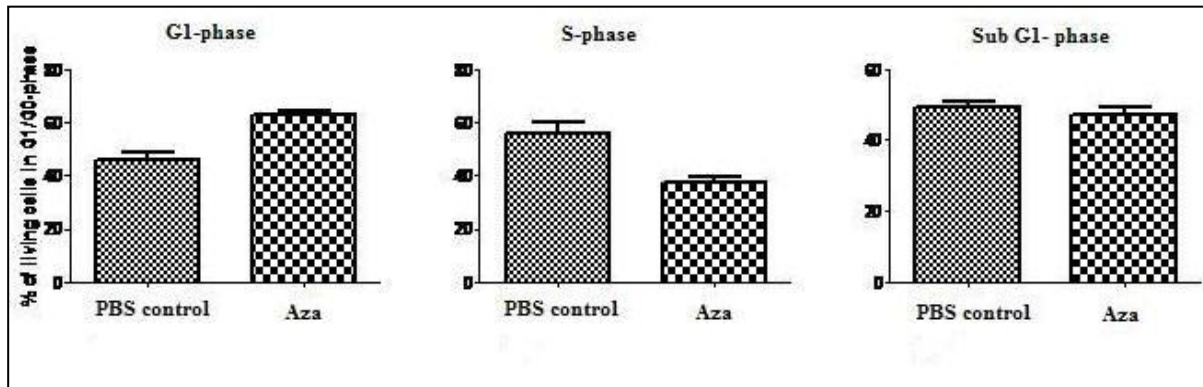


Figure S2 FACS analysis of Aza treated Xenograft tumors. More cells of treated Aza mice are present in the growth G1 phase. In the S-phase due to tumor activity there are more cells present in the untreated (PBS) then in treated mice, whereas Sub G1 phase shows almost same number of nuclei of apoptotic cells in treated and untreated mice.

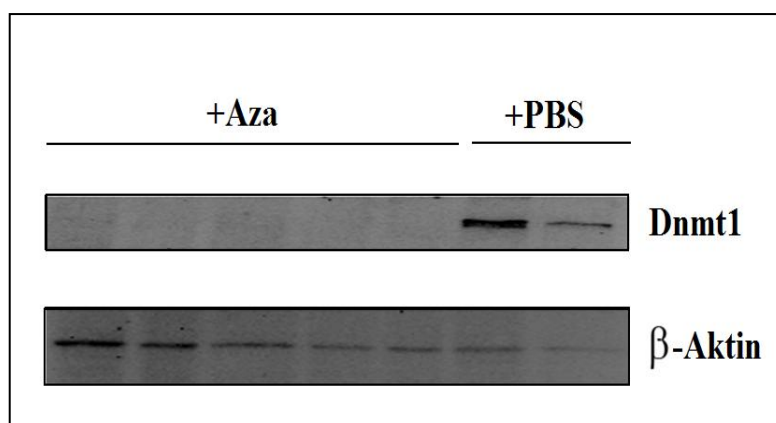


Figure S3 Protein analysis of DNMT1 after treatment with Aza. DNMT1 is depleted in the treated mice compared with the untreated (PBS), where the protein is still expressed.

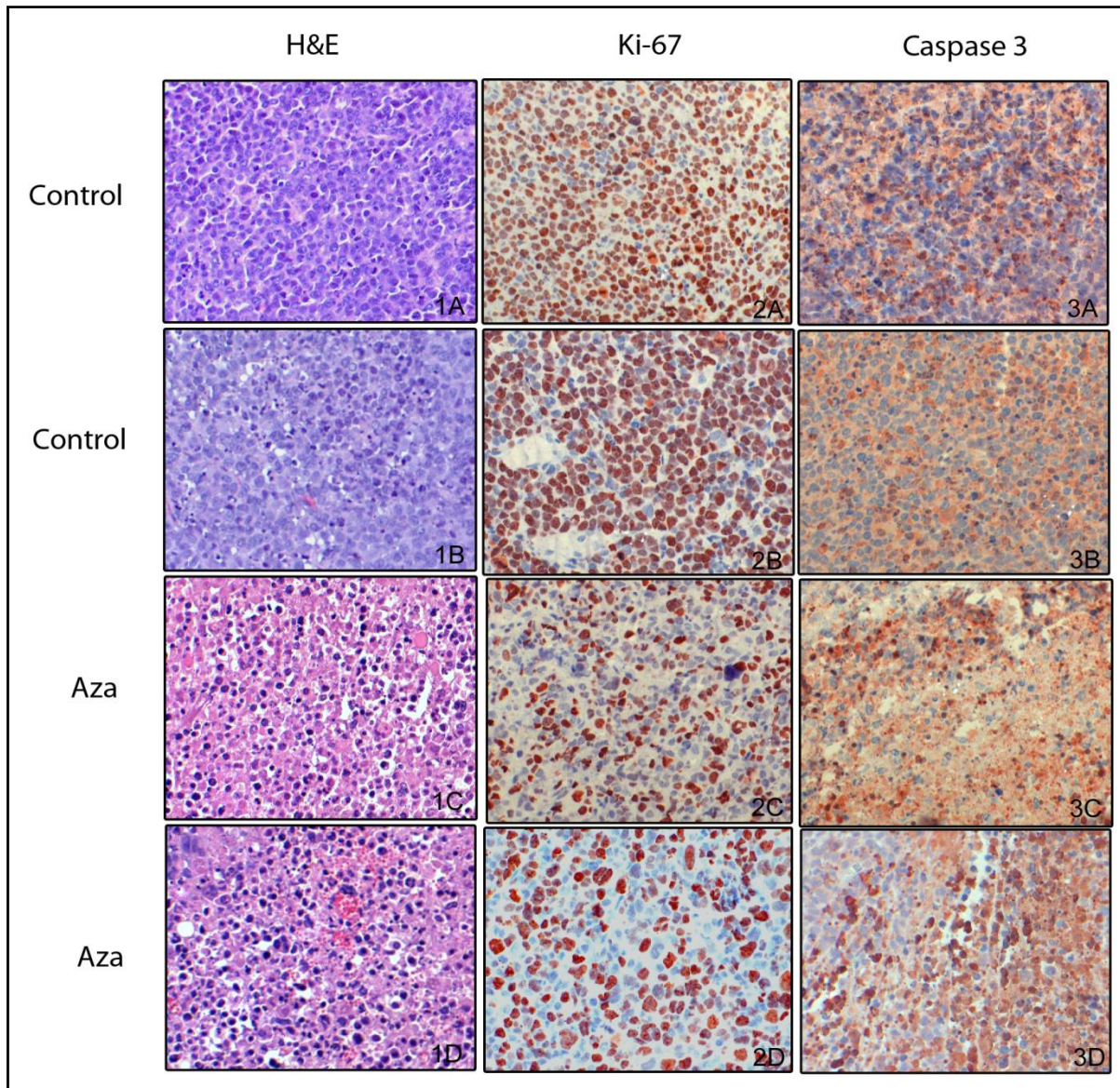


Figure S4 Immunohistochemistry Staining of Aza treated tumor tissues. In Aza treated mice (1C, 1D) there are more apoptotic areas compared to non-treated mice (1A, 1B). Proliferation increases in untreated (2A, 2B) compared with lower proliferation rate in treated mice (2C, 2D). Differences in Caspase 3 expression cannot be observed in the both groups.

7.1.2 Xenograft 2

Aza	Day 0			Day 1			Day 2		
	Tu Lenght	Width	Circ.	Tu Lenght	Width	Circ.	Tu Lenght	Width	Circ
601L	0	0	0	0	0	0	0	0	0
601R	0	0	0	0	0	0	0	0	0
602L	0	0	0	0	0	0	0	0	0
602R	0	0	0	0	0	0	0	0	0
603L	0	0	0	0	0	0	0	0	0
603R	0	0	0	0	0	0	0	0	0
604L	0	0	0	0	0	0	0	0	0
604R	0	0	0	0	0	0	0	0	0
605L	0,65	0,55	0,3575	0	0	0	0	0	0
605R	0	0	0	0	0	0	0	0	0
MITTELWERT			0,03575			0			0
Control	Day 0			Day 1			Day 2		
	Tu Lenght	Width	Circ.	Tu Lenght	Width	Circ.	Tu Lenght	Width	Circ
615L	0,75	0,8	0,6	0,75	0,65	0,4875	0,85	0,75	0,6375
615R	0	0	0	0,8	0,85	0,68	0,8	0,85	0,68
616L	0,6	0,65	0,39	0,55	0,55	0,3025	0,6	0,6	0,36
616R	0	0	0	0	0	0	0	0,5	0
617L	0,75	0,75	0,5625	0,65	0,6	0,39	0,85	0,8	0,68
617R	0,6	0,65	0,39	0,65	0,6	0,39	0,75	0,7	0,525
MITTELWERT			0,32375			0,375			0,48042

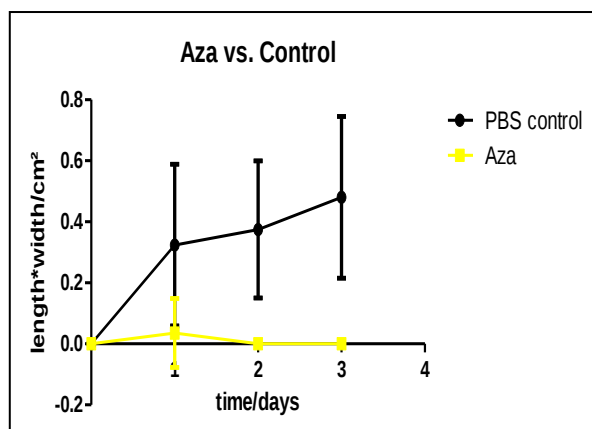
Table S3 Measurements of tumor growth in Aza treated and untreated mice.

No.	Tu Weight	605L	0,027
601R	0,1	MITTELWERT	0,011444444
601L	0	No.	Tu Weight
602R	0	615R C	0,27
602L	0	615L C	0,23
603R	0	616R C	0,24
603L	0	616L C	0,44
604R	0,003	617R C	0,17
604L	0	617L C	0,13
605R	0	MITTELWERT	0,25

Table S4 Tumor weight measurements of Aza treated and control mice.

Control mice (C)

a.



b.

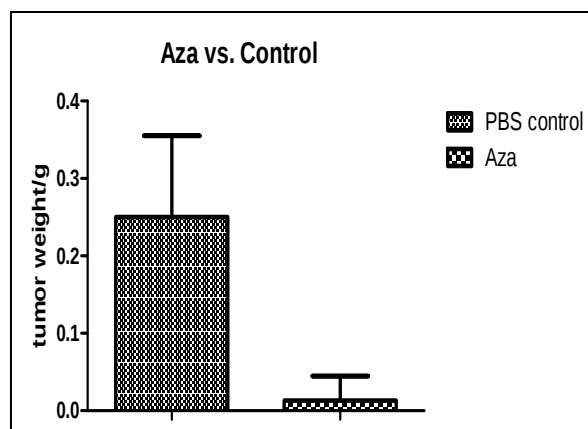


Figure S5 (a) Tumor growth measurement of Aza treated mice. Tumor growth is highly reduced in treated compared with untreated control. **(b)** Tumor weight is heavily decreased in treated mice (n=3) compared with the control group (n=6).

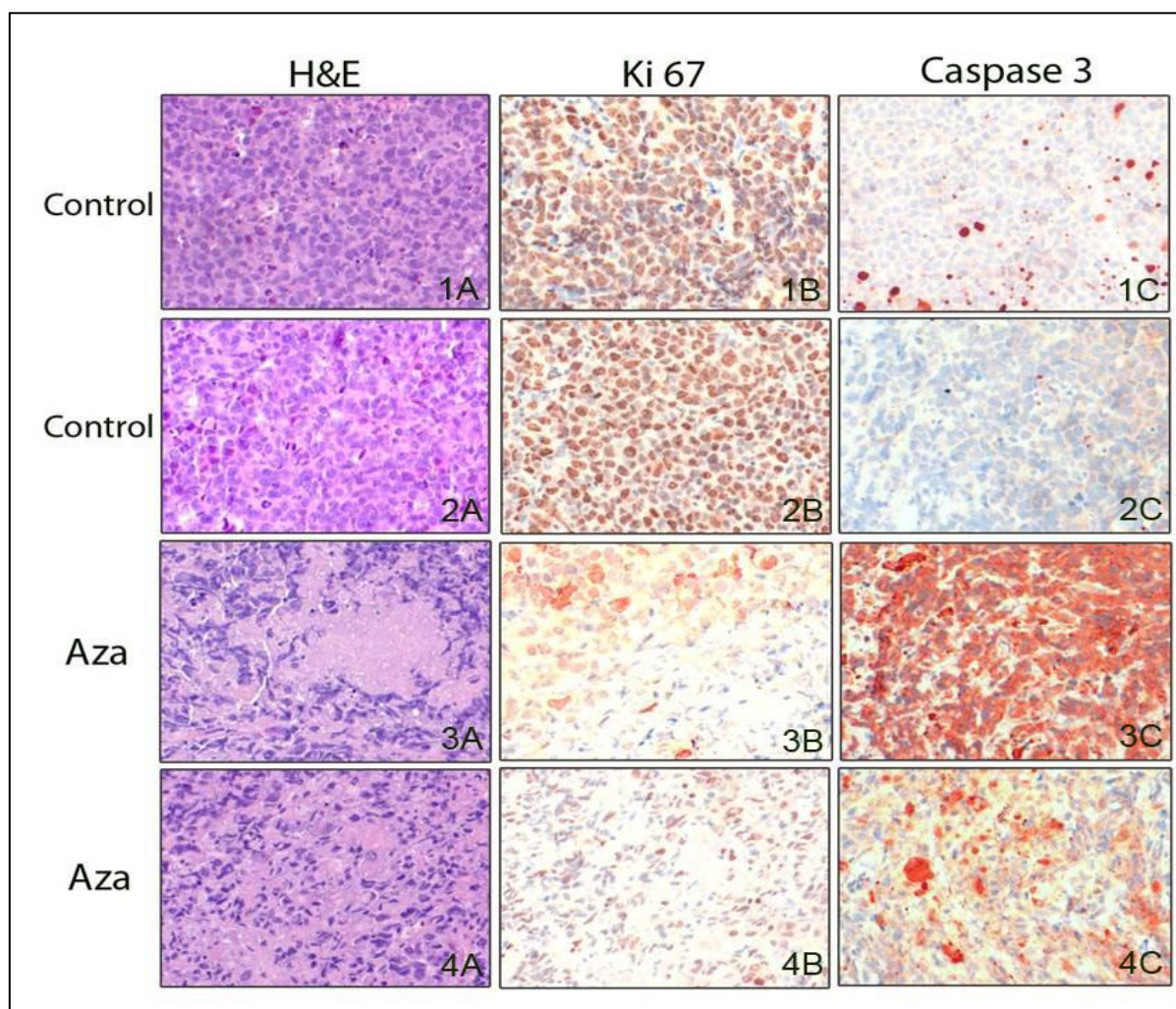


Figure S6 Immunohistochemistry Staining of Aza treated tumor tissue. Increased apoptosis in treated mice (1C, 1D) compared with untreated control group (1A, 1B). Proliferation is also highly increased in untreated mice (2A, 2B) then in treated mice (2C, 2D). Caspase 3 activity is increased in treated (3C, 3D) mice compared with the untreated control group (3A, 3B).

7.1.3 Xenograft 3

	Day 1			Day 3			Day5			Day7		
Aza early	Tu R Lenght	Width	Circ.	Tu R Lenght	Width	Circ.	Tu R Lenght	Width	Circ.	Tu R Lenght	Width	Circ.
f C89 52 R	0	0	0	0	0	0	0	0	0	0	0	0
f C89 52 L	0	0	0	0	0	0	0	0	0	0	0	0
m C89 78 R	0	0	0	0,7	0,65	0,455	0	0	0	0	0	0
m C89 78 L	0	0	0	0	0	0	0,8	0,75	0,6	0,7	0,6	0,42
m C8979/C8	0	0	0	0	0	0	0	0	0	0	0	0
m C8979/C8	0	0	0	0	0	0	0	0	0	0	0	0
MITTELWERT			0			0,0758333			0,1			0,07
	Day 1			Day 3			Day5			Day7		
Aza late	Tu R Lenght	Width	Circ.	Tu R Lenght	Width	Circ.	Tu R Lenght	Width	Circ.	Tu R Lenght	Width	Circ.
f C89 84R	0	0	0	0	0	0	0	0	0	0	0	0
f C89 84 L	0,6	0,6	0,36	0,6	0,6	0,36	0,6	0,8	0,48	0	0	0
m C89 77R	0	0	0	0,8	0,5	0,4	0,8	0,65	0,52	0,9	0,65	0,585
m C89 77L	0	0	0	0,5	0,85	0,425	0,6	1	0,6	0,75	1	0,75
m C89 80 R	0	0	0	0	0	0	0	0	0	0	0	0
m C89 80 L	0	0	0	0	0	0	0,6	0,55	0,33	0,6	0,55	0,33
m C89 81 R	0	0	0	0,7	0,7	0,49	0,8	0,85	0,68	0,85	0,85	0,7225
m C89 81 L	0	0	0	0,6	0,6	0,36	0,65	0,6	0,39	0,65	0,75	0,4875
MITTELWERT			0,045			0,254375			0,375			0,47916667
	Day 1			Day 3			Day5			Day7		
Control	Tu R Lenght	Width	Circ.	Tu R Lenght	Width	Circ.	Tu R Lenght	Width	Circ.	Tu R Lenght	Width	Circ.
f C8951 R	0,65	0,55	0,33	0,7	0,65	0,455	0,95	1	0,95	1,1	1,15	1,265
f C8951 L	0	0	0	0,6	0,6	0,36	0,8	0,8	0,64	0,95	1,1	1,05
m C89 76 R	0	0	0	0,75	0,7	0,525	0,8	0,75	0,6	1	0,85	0,85
m C89 76 L	0	0	0	0,6	0,6	0,36	0,8	0,8	0,64	1	1,05	1,05
m C89 82 R	0	0	0	0	0	0	0	0	0	0	0	0
m C 8982 L	0	0	0	0,9	0,8	0,72	0,95	1,05	0,9975	1,1	1,05	1,155
MITTELWERT			0,055			0,40333333			0,63791667			0,895

Table S5 Tumor growth measurements of Aza treated mice.

Aza Early	Tu Weight L	Tu Weight R	MITTELWERT
C 8978	0,14	0	0,05825
C 8979	0,093	0	
Aza Late			
C 89 77	0,34	0,2	
C 89 80	0,056	0	0,1405
C 89 81	0,137	0,11	
Control			
C 89 51	0,77	0,56	
C 89 76	0,36	0,37	0,486666667
C 89 82	0,86	0	

Table S6 Measurements of tumor weight in Aza treated and control mice.

7.2 MS-275 treatment

7.2.1 Xenograft 1

139 R	0,9	0,75	0,68	0,85	0,8	0,68	1	0,75	0,75	1,1	0,95	1,05
139 L	0,9	1	0,90	1,25	1,15	1,44	1,25	0,95	1,19	1,4	1,1	1,54
141/142 R	0,8	0,85	0,68	1	1	1,00	1,1	1	1,10	1,05	1,2	1,26
141/142 L	1	0,7	0,70	1,1	1,35	1,49	1,05	1,3	1,37	1,05	1,15	1,21
146 R	1,1	0,7	0,77	1	1	1,00	1,2	0,9	1,08	1,4	1	1,40
146 L	0	0	-	0	0	-	0,8	0,7		0,7	0,6	
151/152 R	1,3	1		1,5	1,25		1,3	1,05		1,5	1,05	
151/152 L	0,6	0,85	0,51	0,85	0,75	0,64	0,9	0,85	0,77	1	0,75	0,75
155R	1,1	0,9	0,99	0,9	0,8	0,72	0,9	0,85	0,77	1	0,8	0,80
155L	0,8	0,8	0,64	0,9	0,8	0,72	0,9	0,9	0,81	0,9	0,9	0,81
MITTELWERT			0,65			0,85			0,98			1,10
149 R	0,9	0,7	0,63	1,1	0,9	0,99	1,15	1,4	1,61	1,6	1,25	2,00
149 L	0,8	0,8	0,64	1,3	1,1	1,43	1,1	1	1,10	1,3	1,1	1,43
MITTELWERT			0,64			1,21			1,36			1,72
No	Day 4 Tu Length	Width	Circ.	Day 5 Tu Length	Width	Circ.	Day 6 Tu Length	Width	Circ.	Day7 Tu Length	Width	Circ.
139 R	1	0,95	0,95	1,1	1,05	1,16	1,2	1,2	1,44	1	1,8	1,80
139 L	1,45	1,2	1,74	1,4	1,4	1,96	1,6	1,2	1,92	1,6	1,2	1,92
141/142 R	1,3	1,1	1,43	1,3	1,3	1,69	1,45	1,35	1,96	1,4	1,55	2,17
141/142 L	1	1,2	1,20	1,2	1,3	1,56	1,3	1,2	1,56	1,75	1,85	3,24
146 R	1,45	1,05	1,52	1,45	1,2	1,74	1,4	1,2	1,68	1,9	1,3	2,47
146 L	1,5	1,35		0,8	0,65		1,05	0,9		0,9	0,75	0,68
151/152 R	1,45	1		1,4	1,35		1,55	1,55		2,4	1,95	
151/152 L	0,95	0,85	0,81	1,05	0,95	1,00	1,15	1	1,15	1,2	1,1	1,32
155R	1,1	1	1,10	0,95	0,9	0,86	1	1,2	1,20			
155L	1,05	0,9	0,95	1	1,05	1,05	1,05	1,05	1,10			
MITTELWERT			1,21			1,38			1,50			1,94
149 R	1,6	1,25	2,00	1,75	1,4	2,45	1,8	1,6	2,88	2,1	1,9	3,99
149 L	1,3	1,2	1,56	1,3	1,3	1,69	1,5	1,35	2,03	1,55	1,4	2,17
MITTELWERT			1,78			2,07			2,45			3,08

Table S7 Tumor growth measurements in MS-275 treated mice.

Tumor Weight		
No	Tumor weight/g	
139 R	1,32	
139 L	2,74	
141/142 R	1,23	
141/142 L	2,15	
146 R	2,29	
151/152 L	0,65	
146 L		0,54 excluded because no tumor day0
151/152 R		3,3 excluded because tumor too big on day 0
155R		1,31 excluded because one day less therapy
155L		0,51 excluded because one day less therapy
MITTELWERT	1,73	
149 R	1,91	
149 L	1,38	
148 R	1,85	
148 L	1,95	
MITTELWERT	1,7725	

Table S8 Tumor weight measurements in MS-275 treated mice.

8. Abbreviations

AKTv	akt murine thymoma viral oncogene homolog
ALK	anaplastic lymphoma kinase
APC	adenomatous polyposis coli
ARF	alternate reading frame
CASP3	Caspase 3
CCND1	CYCLIN D1
C-JUN	jun proto-oncogene
DNMT1	DNA (cytosine-5-)-methyltransferase 1
DNMT2	tRNA (cytosine-5-)-methyltransferase 2
DNMT3a	DNA (cytosine-5-)-methyltransferase 3 alpha
DNMT3b	DNA (cytosine-5-)-methyltransferase 3 beta
DNMT3L	DNA (cytosine-5-)-methyltransferase 3-like
ERK	elk-related tyrosine kinase
EZH2	enhancer of zeste homolog 2
GASC1	lysine (K)-specific demethylase 4C
GSTP1	glutathione S-transferase pi 1
IRS1	insulin receptor substrate-1
JAG1	Jagged
JAK2	Janus kinase 2
JAK3	Janus kinase 3
Jmjd3	lysine (K)-specific demethylase 6B
LEF	lymphoid enhancer-binding factor 1

LSD1	lysine (K)-specific demethylase 1A
MAPK	mitogen-activated protein kinase
MYC v	myc myelocytomatosis viral oncogene homolog (avian)
NFATC	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1
NPM1	nucleophosmin
NSD1	nuclear receptor binding SET domain protein 1
p16	cyclin-dependent kinase inhibitor 2A
p21	cyclin-dependent kinase inhibitor 1A
p27	cyclin-dependent kinase inhibitor 1B
p53	tumor protein p53
PI3-K	phosphoinositide-3-kinase, catalytic, gamma polypeptide
PLCγ	phospholipase C, gamma
PTEN	phosphatase and tensin homolog
RASSF1A	Ras association (RalGDS/AF-6) domain family member 1
Rb	Retinoblastoma
SHC	SH2 domain-containing transforming protein
SHP1	protein tyrosine phosphatase, non-receptor type 6
SRC v	src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)
STAT 3	suppressor of cytokine signaling 3
STAT5B	signal transducer and activator of transcription 5B

TCF T-cell factor

WNT2 wingless-type MMTV integration site family member 2

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10. Appendix

CURRICULUM VITAE

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