Efficacy of the HOX-inhibitor HXR9 in acute myeloid leukemia

Master's Thesis in Medicine

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Abstract

Mounting evidence suggests that transcription factors of the HOX family, previously best known for their role in embryogenesis, are involved in several other processes, among them hematopoiesis, and that overexpression can contribute to malignant transformation. Inhibition of HOX functions may be a potential treatment approach for hematopoietic malignancies.

In this study we have investigated the effect of HXR9, a peptide designed to inhibit the interaction between HOX proteins and a cofactor, on acute myeloid leukemia (AML) cell lines *in vitro*. We show that AML-cells enter apoptosis when exposed to HXR9, in addition, the effect is dose-dependent but not specific to HXR9 as the negative control peptide CXR9 is also cytotoxic to the AML-cells, albeit to a lesser extent. Furthermore, we show that long time exposure to HXR9 may induce maturation in AML cells, though our results are inconclusive. Finally, we demonstrate that the cytotoxic efficacy and possibly pro-maturating effect is independent of the level of HOXA9 — a HOX protein commonly overexpressed in AML.

Contents

1 Background

1.1 Hematopoiesis

Hematopoiesis is the formation of blood cells and takes place in the bone marrow. All blood cells derive from hematopoietic stem cells (HSC) [1]. Expansion of the highly proliferative HSC and differentiation via different pathways give rise to erythorcytes, thrombocytes and leukocytes (see figure 1.1).

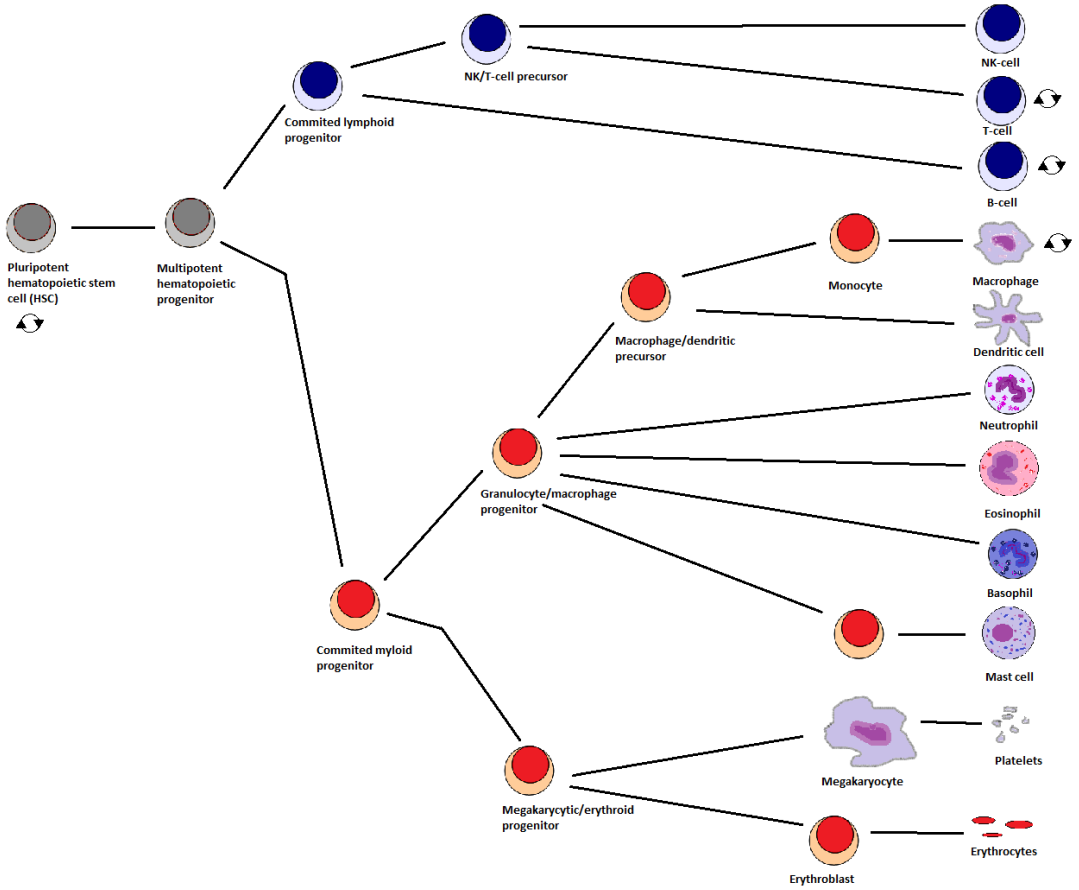


Figure 1.1: Hematopoiesis. Adapted from [1].

1.2 Acute Myeloid Leukemia (AML)

Cancer originating from the blood-forming organs is called leukemia. Depending on which cell line is affected, leukemia can be either lymphatic or myeloid. It can be further classified as acute or chronic, depending on the rate of progression. AML originates from transformed hematopoietic progenitors that have acquired a block in differentiation and leads to the accumulation of dysfunctional immature cells (blasts) of myeloid lineage [2]. The rapid expansion of myeloblasts in bone marrow and blood inhibits the normal hematopoietic function of the bone marrow and untreated AML soon leads to death [2].

1.2.1 Epidemiology

AML primarily affects the elderly. The annual incidence of AML in Sweden is 340 and the median age at diagnosis is 71 years [3]. The overall survival 5 years after diagnosis is 20–30%, highly dependent on the age of the patient and the genetic profile of the malignant cells [3]. In younger patients (0–49 years of age) the 5-year survival rate is approximately 60% [3].

1.2.2 Subclasses

Subgroups within the AML population show clinically relevant differences in prognosis and response to treatment. As an aid in managing with these differences the disease is categorized in subgroups defined by the World Health Organization (WHO) [2]. All available information, including morphology, immunophenotype and genetics, is used for classification [4]. The system was revised in 2008 and among the changes was the recognition of AML with mutated NPM1 as a separate entity (see table 1) [4].

Table 1: The WHO classification of acute myeloid leukemias. Adapted from [4].

Acute myeloid leukemia with recurrent genetic abnormalities
- AML with t(8;21)(q22;q22); RUNX1-RUNX1T1
- AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11
- APL with t(15;17)(q22;q12); PML-RARA
- AML with t(6;9)(p23;q34); DEK-NUP214
- AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); RPN1-EVI1
- AML (megakaryoblastic) with t(1;22)(p13;q13); RBM15-MKL1
- *Provisional entity: AML with mutated NPM1*
- *Provisional entity: AML with mutated CEBPA*

Acute myeloid leukemia with myelodysplasia-related changes

Therapy-related myeloid neoplasms

Acute myeloid leukemia, not otherwise specified
- AML with minimal differentiation
- AML without maturation
- AML with maturation
- Acute myelomonocytic leukemia
- Acute monoblastic/monocytic leukemia
- Acute erythroid leukemia
- Acute megakaryoblastic leukemia
- Acute basophilic leukemia
- Acute panmyelosis with myelofibrosis

Myeloid sarcoma

Myeloid proliferations related to Down's syndrome

Blastic plasmacytoid dendritic cell neoplasm

25–30% of all AML is still classified as "AML, not otherwise specified" but this group continues to shrink as more genetic subgroups are identified [4]. The unspecified cases are divided in groups roughly corresponding to the old FAB (French-American-British) systems eight subclasses according to morphology (see table 1)[5].

1.2.3 Treatment

The current curative treatment for AML starts with chemotherapy. The aim of the first cycle, induction chemotherapy, is to achieve complete remission (CR), defined as microscopical disappearance of cancer cells from blood and bone marrow [2]. Following induction therapy, consolidation therapy is administered whose aim is to eliminate any remaining leukemic cells [2]. Approximately 70% of patients achieve CR [3]. Unfortunately many patients relapse, which significantly explains why the long term survival remains as poor as 20–30% despite the

high rate of CR [3].

In addition to chemotherapy some patients receive allogenic stem cell transplantation [2]. This treatment is associated with significant mortality and morbidity [2], making the choice of whether or not to proceed with a transplantation something to carefully consider. The risk of relapse, the availability of a matching donor and the general condition of the patient are taken into account [2].

In recent years knowledge of the genetic changes and the mechanisms behind AML has increased significantly [4], which is an important step towards identification of new and more specific targets for therapy. However, the prognosis remains grim and there is still need for a better understanding of the mechanisms behind the disease and how these can be exploited in order to improve patient outcome.

1.3 HOX genes and their role in leukemia

HOX genes code for a family of transcription factors that are well known for their role in the developing embryo where they control the head-tail axis and decide segmental identity [1]. The HOX proteins contain a highly conserved peptide sequence, called the homeodomain, that allows for DNA binding [1]. HOX genes are highly conserved across species and time [6], presumably due to their vital role in embryonic developement, where disruption of HOX expression causes malformation [7].

In humans, the HOX genes are found in clusters (A to D) on four different chromosomes [8]. The organization into clusters is believed to be the results of gene duplications of a single cluster of genes [8]. HOX genes from different clusters can be aligned according to homology of their homeobox regions, forming paralog groups, for example HOXA9, HOXB9, HOXC9 and HOXD9 [6]. The gene coding for HOXA9, which is of particular interest in this study, is found in cluster A and is of the paralog group 9.

1.3.1 HOXA9 in hematopoiesis

In addition to their role in developmental biology HOX proteins are also involved in hematopoiesis, among them HOXA9. HOX proteins seem to have a regulating function on hematopoiesis, controlling the proliferation of hematopoietic stem cells (HSCs). This has been demonstrated both by the inability of HOXA9 deficient mice to repopulate bone marrow after irradiation due to reduced proliferation [9] and by the fact that overexpression of HOXA9 induces stem cell expansion [10].

Many HOX genes are strongly expressed in HSCs and early progenitors, but the expression is downregulated in more differentiated cells [11], suggesting a role in differentiation as well as in proliferation. Thorsteinsdottir et al. [10] showed that overexpression of HOXA9 induces a partial block in B lymphopoiesis as well as enhancing myelopoiesis resulting in an increased number of mature granulocytes. It has also been shown that switching certain HOX proteins on or off can change the phenotype of blood cells [12].

1.3.2 HOXA9 in leukemogenesis

HOX proteins are expressed in a higher rate in leukemic cells than in normal hematopoietic cells, where HOX genes normally are downregulated as the cells differentiate [13]. Evidence suggests that especially the posterior HOXA genes, among them HOXA9, are important in leuke

mogenesis [14]. One research group has shown that human HSCs treated to over express HOXA9 were able to establish permanent cell lines [14], and another group reported that overexpression of HOXA9 transformed bone marrow cells in mice, resulting in AML after 7 months [15]. The delay suggests that HOXA9 in itself is not enough to transform the cells, and there is a need for acquisition of further mutations for leuke

mogenesis to occur [10].

1.3.3 Cofactors

All HOX proteins contain the same polypeptide structure for DNA binding, called the homeodomain, and while the homeodomain allows DNA binding it does not explain the unique effects of different HOX proteins, for example the exactness of malformations caused by mutations in or absence of specific HOX genes [1]. However, in addition to the homeodomain, HOX proteins contain sequences that are distinctive for each HOX protein and allow binding to protein partners [1]. Such partners, called cofactors, increase the precision of DNA binding as well as determine whether the activity at the binding site will cause activation or repression [1, 16]. Pre B-cell leukemia transcription factors (PBX) are examples of HOX cofactors, that contain a homeodomain, but are not part of the clustered HOX gene family. Several studies have shown that cofactors are involved in hematopoiesis and that they can enhance the transforming ability of HOX proteins [14, 17].

1.4 Common mutations in AML and their association with HOXA9 overexpression

Overexpression of HOX proteins in general and HOXA9 in particular is common in AML, and this feature has been identified as a contributing factor to malignant transformation. However, as previously mentioned, AML is a genetically diverse disease and there are many pathways and mutations that can cause leukemic transformation. In order to study the importance of HOXA9 overexpression it is necessary to identify which subgroups of AML that have a high expression of HOXA9, and preferably to understand the mechanism behind such an overexpression.

1.4.1 The nucleophosmin (NPM1) mutation in AML

The nucleophosmin (NPM1) gene is mutated in one third of all AML, making it the most common mutation associated with AML [18]. The mutation is always heterozygous, leaving one functional NPM1 wild type allele [19], and is strongly associated with a normal karyotype (normal number, shape and size of chromosomes) [18].

In the 2008 revision of WHO classification of myeloid neoplasms, NPM1 mutated AML was introduced as a separate entity, albeit as a provisional one [4].The following evidence has been forwarded to support that NPM1 mutations define a separate entity of AML; NPM1 mutation excludes other recurrent genetic abnormalities and vice versa [20], AML with mutated NPM1 has a distinctive gene expression signature [21, 22], all NPM1 mutations result in cytoplasmic dislocalization of the protein [18] implicating this as a critical feature.

Normal function of nucleophosmin (NPM) Nucleophosmin (NPM) is a protein expressed in all human tissues and it has several important functions, some of which are: controlling the duplication of centrosomes during mitosis, regulating the response to stress stimuli by modulating the stability of oncosuppressor p53, and growth-suppression through stabilization of oncosuppressor ARF [19]. NPM also plays a vital role in the assembly of ribosomes, thsu contributing to cell proliferation and growth by chaperoning ribosomal RNA from the nucleus to the cytoplasm [19]. To be able to shuttle between the nucleus and the cytoplasm, NPM includes several sequences regulating its position in the cell, which are all located close to the C-terminus [19]. Among them is a short aromatic stretch including 2 tryptophans at positions 288 and 290 that are important for binding to the nucleus [23]. In normal tissue most of the NPM is located in the nucleoli and only a small part is located in the cytoplasm because the nuclear import signals are stronger than the export signals [19].

Cytoplasmic nucleophosmin (NPMc+) In a paper from 2005 Falini et al. [18] described a subgroup of AML patients where NPM is dislocated to the cytoplasm, NPMc+. These authors sequenced the NPM1 gene and identified 6 different mutations, all in exon 12 [18]. The dislocation of the protein is explained by alterations at the C-terminus of the gene, which disrupts the aromatic stretch including tryptophans at positions 288 and 290 [18], reducing the nuclear binding capacity of the protein, while simultaneously introducing another nuclear export signal (NES) [24, 25]. Together this shifts the balance so that most of the mutated NPM dislocates to the cytoplasm [25]. The mutated NPM then interferes with wild type NPM protein, binding it to the cytoplasm as well [25].

The mechanism by which the mutation causes leukemia is still not clear. However, the mutation has only been found in AML cells [18, 26], strongly suggesting a causal relation. As previously mentioned, wild-type NPM acts as a oncosuppressor, which means that mutations involving NPM1 could be oncogenic both through the reduced expression and function of wild-type NPM (loss of function) and through the function of the mutated NPM (gain of function).

Association between NPMc+ and overexpression of HOXA9 Analysis of NPM-mutated AML cells showed a distinct pattern of gene expression [22]. Several HOX genes and cofactors are up-regulated, among them are HOXA9 and PBX3 [22]. The mechanism by which NPMc+ induces overexpression of these proteins is not known. Nevertheless, HOX genes and HOXA9 in particular have been prpposed to induce leukemia, as described in subsection 1.3.2, and thus upregulation of HOX genes could be part of the mechanism behind NPMc+ AML.

1.4.2 Mixed lineage leukemia (MLL) and the MLL-gene

The MLL gene was identified while searching for the gene involved in translocations found to be present in a group of infants with leukemia sharing the same clinical profile and poor prognosis [27]. The hematopoietic blasts of these patients showed signs of both lymphatic and myeloid differentiation and while some of the patients were diagnosed with AML, others were diagnosed with Acute Lymphatic Leukemia (ALL) and some even experienced a lineage switch during the course of the disease [27]. Thus, the gene was named Mixed lineage leukemia (MLL).

MLL regulates gene expression The MLL gene codes for a histone methyltransferase, an enzyme able to methylate histone, allowing DNA to unravel and become available for transcription. Among the genes targeted and regulated by MLL is the HOX family [28].

Studies have shown that after transcription of the MLL gene, the protein is cut in two pieces, N-terminal MLL-N and C-terminal MLL-C [29]. Although cut into separate parts they associate in the same complex, the MLL complex, where they have different roles. MLL-N is involved in the targeting and binding to DNA while the MLL-C contains and associates to structures

important in regulating the transcription of targeted DNA, including chromatin modification [27].

MLL translocations cause HOX overexpression MLL translocations fuse the MLL N-terminal to a partner gene [28, 30]. The fusion protein thus shares the N-terminal part with wild type MLL, while the MLL C-terminal is switched out. To better understand the function of MLL fusion proteins, studies of the fusion partners have been performed. As of yet, 66 fusion partners have been identified and one of the common partners is AF9 [31]. Studies of the fusion partners have showed that they are involved in the control of transcription elongation and that several of them associate in the same complex[27].

Based on these results, it seems reasonable to assume that the MLL fusion protein would combine binding to MLL wild type target loci (including HOX genes) with transcription elongation, resulting in a upregulation of the target genes. It has been confirmed that MLL-rearranged AML cells express high levels of HOX genes [32], and this upregulation is considered to be a major mechanism by which MLL fusion proteins cause leukemia [28]. The theory is further supported by a study which reported that inhibition of HOXA9 using the peptide HXR9, described in subsection1.5.1, leads to apoptosis of MLL cells [17].

1.4.3 DNA Methyltranferase 3A (DNMT3A)

DNMT3A is an enzyme catalyzing DNA methylation, a process where a methyl group is added to a DNA nucleotide. DNA methylation is important in the regulation of gene expression by inhibition of gene transcription. In a study from 2014, Qu et al. [33] showed that AML cells expressing a mutated form of DNMT3A exhibit global DNA hypomethylation and statistically significant hypomethylation of HOX genes, implicating an increased expression of these HOX genes.

1.4.4 The Myc oncogene

The Myc protein is a multifunctional protein and an important oncoprotein in human cancer. Myc acts as a transcription factor that promotes growth and is involved in the clocking of the cell cycle [34]. In 15–20% of human cancers the *Myc* gene is deregulated, resulting in overexpression of the Myc protein, which then operates as an oncoprotein promoting excessive growth and transformation to cancer [34]. There is no known association between *Myc* mutations and HOXA9 over expression.

1.5 Inhibition of HOX proteins

With evidence lining up to support the hypothesis that HOX proteins play a role in the transformation of hematopoietic cells, it has become interesting to study the effect of HOX inhibition. There are many ways to inhibit HOX function, one of which is to target the interaction between HOX proteins and their cofactors. In a study from 2013 Li et al [17] shows that the cofactor PBX3 is important for leukemic transformation and that inhibition of the interaction between PBX3 and its HOX partners promotes apoptosis in AML cells.

1.5.1 The HOX inhibitor HXR9

HXR9 was designed by Morgan et al [35] with the intention of creating a peptide that specifically blocks the interaction between HOX transcription factors and PBX cofactors. In this study it will be used to study the importance of the interaction between HOXA9 and PBX3.

HXR9 consists of 18 amino acids and contains a hexapeptide sequence (YPWM) and a polyarginine sequence (R9), see figure 1.2. The YPWM hexapeptide is present in many HOX

HXR9 W**YPWM**KKHHRRRRRRRRR

CXR9 W**YPAM**KKHHRRRRRRRRR

Figure 1.2: Peptide sequences for HXR9 and CXR9. The hexapeptide sequence is in bold and the substituted amino acid is underlined.

proteins from group 1–9 and is required for their ability to bind to PBX [16, 36]. The polyarginine sequence of 9 consecutive arginine residues is a cell-penetrating peptide that allows delivery into mammalian cells without the need for any specific receptors [37].

CXR9 is identical to HXR9 except for a single substituted amino acid (figure 1.2). The substitution disrupts the hexapeptide at a critical point, so CXR9 should not be able to bind to PBX [16, 36]. CXR9 still carries the polyarginine sequence, which will allow it to enter the cell and thus work as a negative control.

Previous studies have shown a cytotoxic effect of HXR9 on several different types of cancer cells, among them melanoma [35], meningioma [38] prostate cancer [39], and breast cancer [40]. To date, there is only one published study of HXR9 and AML, and it shows that HXR9 causes apoptosis in AML-cells and that the efficacy is dependent on the level of expression of HOX-genes [17], in accordance with the proposed mechanism of action.

2 Aim

The overall aim of this study is to determine the effect of the HOX inhibitor HXR9 on AML cells with different mutations. More specifically:

- Does HXR9 stimulation of AML cells induce apoptosis?
- Can HXR9 stimulation over a longer period of time induce differentiation in AML cells, as defined by expression of myeloid markers or a reduced capacity to form colonies?
- Is the effect of HXR9 dependent on HOXA9 expression level?

3 Method

3.1 Cell cultures

Four human AML cell lines and one murine liquid culture were used in the experiments. The human cell lines were cultured in Iscoves medium with 10% heat-inactivated fetal calf serum (FCS), 1% penicillin and streptomycin, 1% L-glutamine, and 1% Na-pyruvate. For the murine derived liquid culture a DMEM medium with 20% fetal calf serum, 1% penicillin and streptomycin, 1% L-glutamine, 50 pg/ml mouse SCF (stem cell factor), 10 pg/ml human IL-6, and 6 pg/ml mouse IL-3 was used. Passaging of the cells was performed three times a week. A description of the cell lines follws:

3.1.1 HL-60

The HL-60 cell line was established from a 36-year old female and carries a mutation in the Myc gene [41]. In a study by Li et al it was shown that HL-60 has a low expression of HOXA9 and that the cells are not affected by stimulation with HXR9/CXR9 and is therefore used as a negative control [17].

3.1.2 THP-1

According to the distributor ATCC the THP-1 cell line was established from a 1 year old male infant [42]. THP-1 cells have the translocation t(9:11) resulting in the MLL-AF9 fusion gene [43]. MLL fusion proteins are known to induce overexpression of HOX proteins (see subsection 1.4.2) and this particular cell line has been shown to become apoptotic in the presence of HXR9 [17], making it suitable as a positive control in this study.

3.1.3 OCI-AML2

According to the distributor Leibniz-Institut DSMZ the cell line was *"established from the peripheral blood of a 65-year-old man with acute myeloid leukemia (AML FAB M4) in 1986 at diagnosis"*[44]. This cell line carries a DNMT3A mutation but no mutation in NPM1.

3.1.4 OCI-AML3

According to the distributor Leibniz-Institut DSMZ the cell line was: *"established from the peripheral blood of a 57-year-old man with acute myeloid leukemia (AML FAB M4) at diagnosis in 1987"* [44] and *"cells carry an NPM1 gene mutation (type A) and the DNMT3A R882C mutation"* [44].

3.1.5 HOXA9

This liquid culture was established by Hanna Grauers Wiktorin at Sahlgrenska Cancer Center using murine bone marrow cells which were transfected with an E86-HOXA9 producing retrovirus to express human HOXA9. The transfected cells are tagged with green flourescent protein (GFP).

3.2 Western blot

The Western blot method is used to examine the protein expression of cells. Gel electrophoresis of cell lysates separates the proteins according to size. From the gel the proteins are blotted onto a membrane and stained with primary and secondary antibodies. The secondary antibodies carry horse radish protein (HRP), which produces chemiluminescence when HRP-substrate is available. The chemiluminescence can be detected by a camera, resulting in a photograph where only the stained proteins are visible.

3.2.1 Analysis of the expression of HOXA9, NPM wild type and mutated NPM

In this project the cell lines OCI-AML3, OCI-AML2, THP-1, HOXA9 and HL60 were analyzed for the expression of HOXA9, NPM wild type and NPM mutation A in order to confirm the characteristics of each cell line.

Approximately 5 million cells of each cell line were harvested, spun down and then re-suspended in 1 ml of cold phosphate buffered saline (PBS). The samples were transferred to transparent 2 ml eppendorf tubes and centrifuged again. NuPAGE LDS buffer was prepared as

follows: 1300 ul RNAse free water was mixed with 500 ul of NuPAGE LDS buffer 4x (Life Technologies) and 200 ul of dithiothreitol. After centrifugation the supernatant was removed and the cell pellets were carefully resuspended in 200 ul of the prepared buffer. The samples were then sonicated for 10 minutes, heated to 75ºC for 5 minutes and finally centrifuged. As the samples were still not sufficiently denatured they were sonicated again for 10 minutes, after which they were set for loading on the gel.

NuPAGE 10% Bis-Tris-Gels from Life Technologies were used. Four gels were prepared; one for HOXA9, one for NPM wild type, one for mutated NPM and one for β-actin, used as a loading control. To each gel 15 ul of ladder (SeeBlue Plus2 Prestained Standard, Life Technologies) and 20 ul of each cell line were loaded. The gels were placed in the electrophoresis chambers (XCell SureLock Mini-Cell, Life Technologies) that had been prepared with MOPS SDS running buffer (Life Technologies). Electrophoresis was performed according to manufacturer's instruction, after which the gels were transferred to containers with methanol. The gels were loaded in two blotting machines (TransBlot SD, BioRad) along with Nitrocellulose Blotting Membranes (Life Technologies) and filters soaked in methanol. Blotting was performed according to manufacture'rs instruction. After blotting the membranes were washed in tap water several times. Panceau S dye was applied to the membranes, to confirm that proteins have been blotted onto the membranes. The membranes were washed in methanol to remove the dye, followed by TBST (tris buffered saline with tween) to wash away the methanol. They were then incubated in milk buffer (Blotting Grade Buffer, BioRad) for 1 hour on a rotary shaker. After 1 hour of incubation the membranes were put in individual sealed plastic bags along with a milk buffer containing one of the primary antibodies. The bags were then fastened to a rocking plate in a cold room and incubated over night.

The next day the membranes were washed 3 times for 10 minutes in TBST on a rotating shaker. After washing, the membranes were put in new plastic bags along with a milk buffer containing the secondary antibody, carrying HRP. The plastic bags were taped onto a rocking plate and left to incubate for 1 hour in room temperature. After the secondary staining the membranes were once again washed in TBST, 3 times for 10 minutes, on a rotating shaker.

For development, Thermo Scientific Pierce Fast Western Blot Kits, SuperSignal West Dura was applied according to manufacturer's instructions except for mutated NPM, where Thermo Scientific Pierce Fast Western Blot Kit, SuperSignal West Femto was used. Pictures were taken using a ChemiDoc MP System (BioRad).

Table 2: Antibodies used for Western blot

ANTIGEN	CLONE	MANUFACTURER
β-actin	*unknown*	Cell Singaling
HOXA9	Polyclonal	Millipore
NPM1 wt	EP1848Y	Abcam
NPM1 mut A	Polyclonal	Abcam
Anti-rabbit-HRP	Polyclonal	Cell Singaling

3.3 The peptides HXR9 and CXR9

The peptides were bought and delivered by Biosynthesis with a guaranteed purity of 85%. Peptides were dissolved in water and stored as aliquots at -80ºC.

3.4 Alamar Blue assay

The assay was used to examine cell viability by measuring the metabolic activity of cells. Alamar Blue Cell Viability Reagent (Life Technologies) reagent is added at 1:10 to a sample containing cells. The cells metabolize the reagent, causing it to shift color from blue to red. A plate reader (FLUOstar Omega, BMG Labtech) provides measurement of the absorbance of two wavelengths and the data are analyzed using an equation provided by the datasheet (see appendix A.1) to determine how much of the reagent has been metabolized, which corresponds to the viability/number of cells. For cytotoxicity assays, the procedure is identical, except that the data are analyzed using another equation (see appendix A.1) to determine the ratio of untreated/viable cells compared to a positive growth control.

3.4.1 Optimization of cell counts and incubation time

In order to get a reading within range and thus be able to detect differences in cell viability for the cytotoxicity assay, cell counts and incubation time must be optimized. Triplicate samples consisting of different number of cells dissolved in 100 ul of medium each were planted in flat-bottomed 6 well plates. The surrounding wells were filled with PBS (phosphate buffered saline), to protect from evaporation. After seeding the cells were allowed to recover for 20 hours and then another 20 hours (corresponding to the time for peptide stimulation). After 40 hours the Alamar Blue reagent was added and the cell viability was assessed at different time points, as previously described in subsection 3.4.

3.4.2 Cytotoxicity assay

Samples consisting of 50,000/75,000/100,000 cells (see subsection 5.2) dissolved in 100 ul of medium each were used and all samples were triplicated (figure 3.1). The samples were seeded in flat-bottomed 96 well plates with PBS in the surrounding wells. A triplicate of medium blank samples (without cells) was also added to the plate. After seeding, the cells were allowed to recover for 20 hours, after which the peptide HXR9 was added resulting in concentrations of 0, 1, 5, 10, 15, 20, 30, 40 uM. CXR9 was used as a negative control and was added at the same concentrations. After 20 hours of peptide stimulation, the Alamar Blue reagent was added followed by four hours of incubation (24 hours of peptide stimulation) after which relative cell viability was assessed, as previously explained in subsection 3.4.

Conc. (uM)	0	1	5	10	15	20	30	40	Blank
HXR9									
HXR9									
HXR9									
CXR9									
CXR9									
CXR9									

Figure 3.1: Plate set-up for peptide stimulation for the Alamar Blue cytotoxicity assay. Each well except those in the blank column contain 50,000/75,000/100,000 cells dissolved in 100 ul of medium in addition to the indicated peptide.

3.5 Flow cytometry

Flow cytometry is a method for sorting or analyzing cells with the aid of antibodies or other proteins that can bind to cell structures carrying fluorochromes. A fluorochrome is a chemical compound that emits electromagnetic radiation at a specific wavelength upon absorption of light.

Flow cytometry data are presented as a dot plot where each dot represents one cell. In addition to the fluorescence provided by staining, the cells are discriminated by the results of a forward scatter (FSC) and side scatter (SSC) analysis, which corresponds to the cell size and structure/granularity, respectively.

3.5.1 Annexin V apoptosis assay

In this assay the Invitrogen Annexin V-FITC Apoptosis Detection kit is used. It contains Annexin V-FITC, which binds to phosphatidylserine exposed on apoptotic cells, and ToPro which binds to DNA and is used as a general marker for cell death. Together they allow discrimintation between live, apoptotic and necrotic cells (see figure 3.2). As the HOXA9 cells already carry a GFP-tag (see subsection 3.1.5) another set of antibodies was used for this cell line; Annexin V-Cy5.5 (BD Biosciences) and 7AAD (BD Biosciences). Cy5.5 is just another fluorochrome and 7AAD is a live-dead marker, similar to ToPro, but fluorescent at another wave-length.

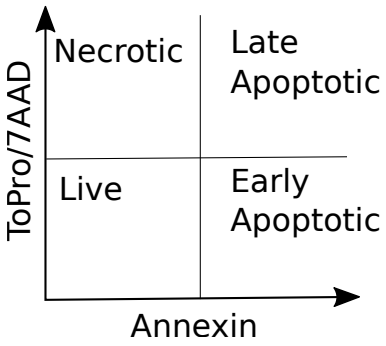


Figure 3.2: Expected distribution of cells in the annexin V apoptosis assay.

For the assay, duplicate samples consisting of 100,000/75,000/50,000 cells (see subsection 5.2) suspended in 100 ul of medium each were seeded in a flat -bottomed 96 well plate, with PBS in the surrounding wells, and allowed to recover for 20 hours. The peptides HXR9 and CXR9 were then added to give final concentrations of 10 uM and 20 uM while one sample

was kept as a negative control (no peptide). After 24 hours of peptide stimulation the paired samples were pooled and washed twice in PBS. After washing, the cells were dissolved in 100 ul of Annexin V-buffer mixed with 2 ul of Annexin V-FITC and 2 ul of ToPro3 or 1 ul of 7AAD. The samples were then incubated in the dark for 10 minutes at room temperature and then diluted with 150 ul Annexin V-buffer before being ready for analysis in the flow cytometry equipment, LSRFortessa (BD Biosciences).

3.5.2 Maturation assay

The aim of this assay was to see if cells cultured in the presence of HXR9 mature, as defined by the expression of myeloid markers. Two panels were designed, one for human and one for murine cells, see table 3.

Table 3: Antibodies used for the maturataion assay. For the human panel the human antibodies and DAPI were used. For the murine panel the murine antibodies were used along with DAPI and GFP.

ANTIGEN	FLUOROCHROME	REACTS WITH	FINAL CONCENTRATION	CLONE	MANUFACTURER
CD11b	PE	Human	1:20	ICRF44	BD Biosciences
CD14	APC-Cy7	Human	1:40	Mφ-P9	BD Biosciences
CD11b	APC-Cy7	Mouse	1:400	M1/70	BD Biosciences
Gr1	PE	Mouse	1:1000	RB6-8CF	BD Biosciences
F4/80	APC	Mouse	1:50	MF48021	Invitrogen
I-A/I-E	PerCP-Cy5.5	Mouse	1:100	M5/114.15.2	BD Biosciences
(nuclear acid dye)	DAPI	Human, mouse	1:3	*N/A*	*unknown*
(cell tag)	GFP	*N/A*	*N/A*	*N/A*	*N/A*

Cells were cultured and passaged as usual for seven days with the addition of one of HXR9, CXR9, DMSO (positive control) or nothing. Cells were seeded in a flat-bottomed 48 well plate using 1 ml samples at normal passaging concentrations, depending on cell line. The peptides and DMSO were added at seeding and passaging: The peptide concentration was 10 uM for HL60, OCI-AML2, OCI-AML3 and HOXA9 and 5 uM for THP-1. The DMSO concentration was 0.25% for HOXA9, 0.5% for OCI-AML2 and OCI-AML3 and 1% for HL60 and THP-1. After seven days the cells were analyzed using the flow cytometry equipment, LSRFortessa (BD

Biosciences), to characterize the expression of myeloid markers.

In preparation for cytometry analysis, the cells were transferred from the plate to tubes and washed twice in E-buffer (PBS with 2 mM EDTA and 0,5% Bovine Serum Albumine). Antibody mixtures were prepared for each panel using E-buffer and appropriate concentration of each antibody. After washing, the cells were transferred to a round-bottomed 96 well plate. The murine cell samples were suspended in 25 ul of a Fc-block and incubated for 5 minutes in room temperature. After incubation, both the human and murine cells were suspended in 50 ul of the appropriate antibody mixture and then incubated in at 4ºC for 30 minutes. After incubation the cells were washed twice and suspended in a 133 ul E-buffer. Immediately prior to the analysis 67 ul of DAPI 3x was added.

3.6 Colony Forming Units (CFU) assay

Hematopoietic stem cells and precursors have the ability to form colonies when cultured in a semisolid medium containing growth promoting and pro-differentiating cytokines. Each colony derives from a single cell, which means that all the cells in a colony are clones. It is possible to distinguish between different types of colonies, corresponding to the different hematopoietic progenitors, depending on the appearance of the colonies. Granulocyte colonies (CFU-G) appear dense and small, with few satellite cells [45]. Macrophage colonies (CFU-M) are sparser but cover a larger area [45]. The appearance of granulocyte-macrophage colonies (CFU-GM) is a mixture of CFU-G and CFU-M. [45]

As cells differentiate they tend to lose their ability to proliferate. By measuring the number of colonies that form in comparison to the number of cells that were plated, the CFU assay provides a measure of the degree of differentiation of the cells cultured. The number of replatings provides another measure of the cells' ability to proliferate. As more and more of the cells differentiate over time, fewer and fewer of them will be able to proliferate, eventually making replating impossible. Replating can also help selecting the cells that are able to form colonies, resulting in more colonies after a one or a few replatings. Normally at least four replatings are performed to assess maturation of cells.

3.6.1 CFU Assay with HL60 and OCI-AML3

The aim of this assay is to see if HXR9 can make leukemic cells lose their stem cell-like ability to proliferate, as defined by a reduced capacity to form colonies. Only OCI-AML3, carrying the NPM1 mutation, and the negative control cell line HL60 is included in this experiment.

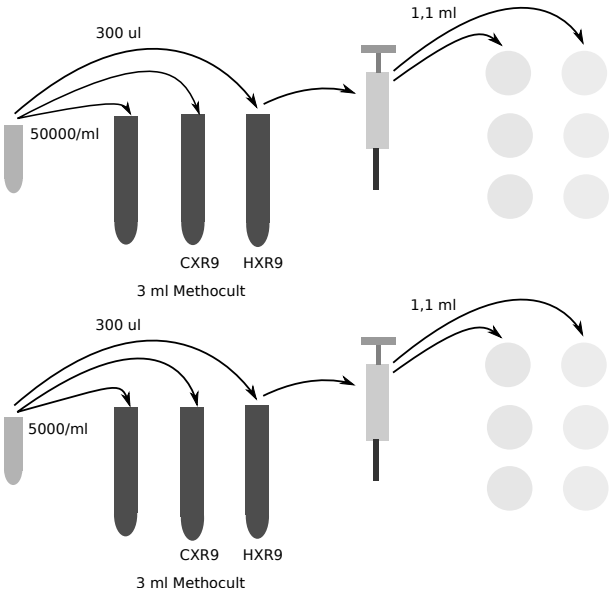


Figure 3.3: Lay-out for the plating of CFUs.

A MethoCult H84534 (StemCell Technologies) semisolid medium was used, containing methylcellulose in Iscoves medium, fetal bovine serum, bovine serum albumin, 2-mercaptoethanol, recombinant human stem cell factor, recombinant human GM-CSF, recombinant human G-CSF, and recombinant human IL-3.Cells were taken from culture and diluted to 500,000 cells/ml in Iscoves' medium without any additives. This solution was then diluted 1:10 to 50,000 cells/ml and again 1:10 to 5,000 cells/ml, see figure 3.3. From these two solutions 300 ul was added to a prepared vial of 3 ml MethoCult H84534 (Stem Cell Technologies), resulting in 5000 cells/ml and 500 cells/ml. 3 vials of Methocult was prepared for each cell line and each cell concentration. HXR9 and CXR9 were added to one of the tubes each, to a final concentration of 10 uM, while one tube was kept as a negative control. The content of each tube was transferred to two 35 mm petri dishes, 1.1 ml for each dish, using a 5 ml syringe with a blunt needle, resulting in 5 000 cells/plate and 500 cells/plate. The lower concentration was intended for counting and the higher concentration for replating. The paired petri dishes were placed in a 100 mm petri dish together with an open 35 mm petri dish containing distilled water, to prevent the methocult from drying out.

After 6–8 days of incubation in 37 °C and 5% CO2, colonies were counted on the plates seeded with 500 cells/plate, with the aid of a microscope and a gridded petri dish.

One day after counting the cells were replated. In order to dissolve the methocult, PBS

was added and the methocult was scraped from the petri dishes and tranferred to a 50 ml tube. The procedure was repeated until all methocult had been scraped off. PBS was added to a final volume of 30 ml and the liquid was mixed using a pipette and then centrifuged.

After centrifugation the supernatant was removed, leaving 1–2 ml of PBS. PBS was then added to 20 ml volume, mixed and then centrifuged again. The washing was repeated once more before the cells were counted and then plated, as previously described.

3.7 Statistical analyses and graphs

FlowJo V10 has been used for analysis of flow cytometry data and creation of dot plots. Creation of graphs and statistical analyses (Student's t-test) have been performed using GraphPad Prism 6.

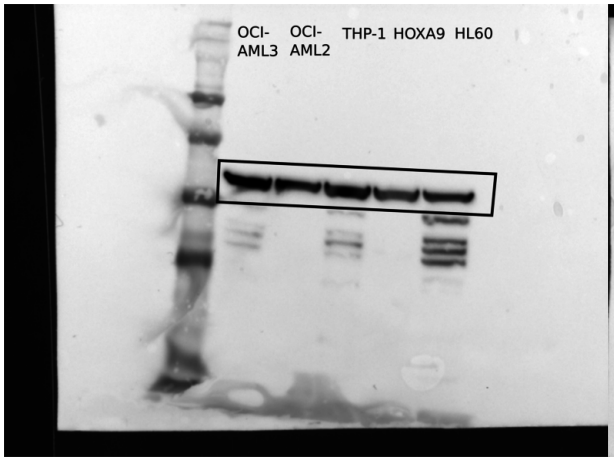
4 Ethics

The experiments involving laboratory mice performed during the establishment of the HOXA9 liquid culture were approved by the applicable committee. All other experiments have been performed *in vitro* using established cell lines.

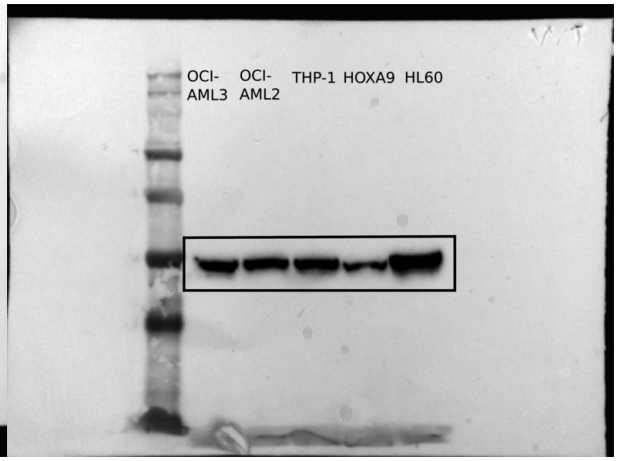
5 Results

5.1 Characterization of protein expression

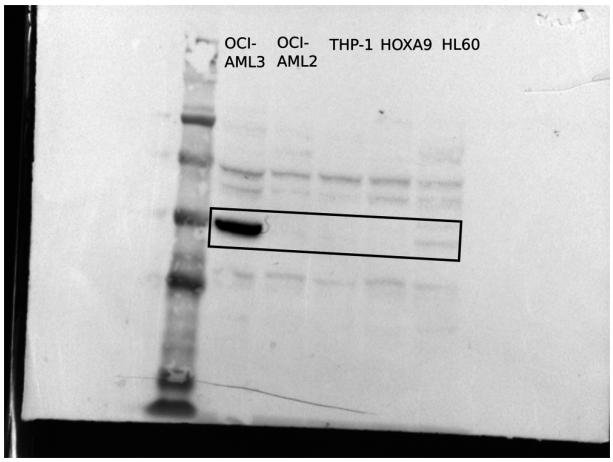
In initial experiments, the expression of NPM1, mutated NPM1 and HOXA9 was determined. Results from the Western blot showed that: all cell lines expressed wild-type NPM (see figure 5.1b), only OCI-AML3 expressed the mutated version of NPM (see figure 5.1c), and THP-1, OCI-AML2, OCI-AML3 had a high expression of HOXA9 while the HOXA9 liquid culture showed an even higher expression (see figure 5.1d). HL60 had a very low expression of HOXA9.



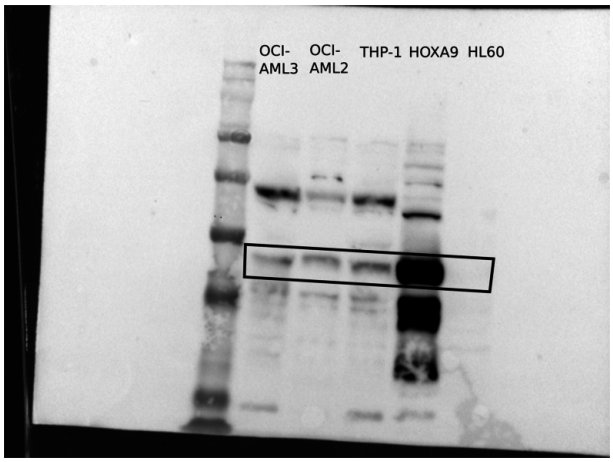
(a) β-actin, loading control.



(b) Wild-type nucleophosmin



(c) Nucleophosmin mutation A



(d) HOXA9

Figure 5.1: Western blot

5.2 Peptide induced cytotoxicity

To investigate the effect of HXR9 an Alamar Blue cytotoxicity assay was set up and performed. Initial test runs showed that with an incubation time of 4 hours, cell counts of 100,000 for HOXA9, 75,000 for HL60, OCI-AML2 and OCI-AML3 and 50,000 for THP-1 were chosen when using 100 ul samples (figure A.1). As many samples were needed, samples with a small volume were desirable.

Data from three independent experiments with triplicate samples showed that all the examined cell lines expressed a reduced viability after exposure to HXR9, as can be seen in figure 5.2. The cells also appeared to be sensitive to CXR9, albeit to a lower extent. The effect of HXR9/CXR9 became significant at 15/40 uM for HL60, 10/15 uM for THP-1, 5/10 uM for OCI-AML2, 30/20 uM for OCI-AML3, and 40/- for HOXA9. The effect of the CXR9 on HOXA9 cells may be significant, but more data is needed as the measured variability is too high.

The specific effect of HXR9 has been calculated as the difference in viability between exposure to HXR9 and CXR9 at a given concentration. The Student's t-test was performed in order to determine the significance of this specific effect. For THP-1 and OCI-AML2 the specific effect was significant at middling concentrations, but at higher concentrations the curves for CXR9 and HXR9 seem be about to converge (see figure 5.2). For OCI-AML3 the specific effect became significant at 30 uM and increased at higher concentrations. For HL60 the specific effect was significant only at 40 uM. For HOXA9 the specific effect was not significant at any of the concentrations examined, due to a high variability between experiments.

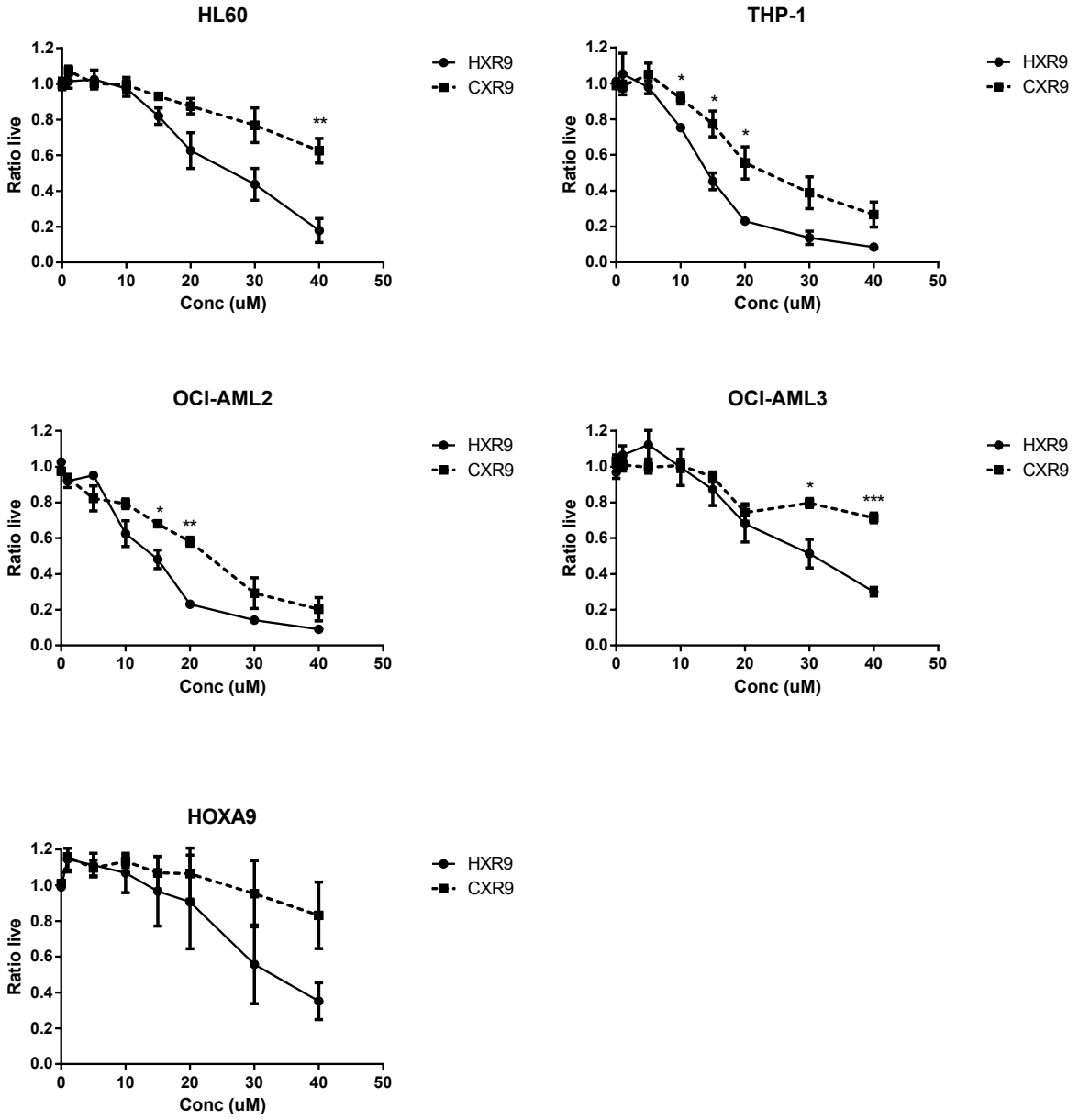


Figure 5.2: Alamar Blue cytotoxicity assay. Cell viability after 24 hours of HXR9/CXR9 peptide stimulation. Mean values and SEM from three independent experiments are shown. One, two, or three * symbols denote a significant difference between HXR9 and CXR9 at the indicated concentrations, according to a Student's t-test with a p-value of <0.05, 0.01, and 0.005, respectively.

To further investigate the cytotoxic effect of HXR9 and to determine if the reduced viability seen in the Alamar Blue cytotoxicity assay (see subsection 5.2) was due to induction of apoptosis, a flow cytometry assay using annexin V and a live-dead marker was performed. Cells were treated with CXR9 and HXR9 for 24 hours followed by flow cytometry to measure the ratio of apoptotic, necrotic and live cells. Representative dots plots from one of three independent experiments are shown in figure 5.3.

The data showed that all the cell lines examined die when exposed to HXR9, and to a lesser extent when exposed to CXR9. The effect of HXR9/CXR9 became significant at 10/10 uM for HL60, 10/20 uM for THP-1, 10/10 uM for OCI-AML2, 10/20 uM for OCI-AML3 and 10/- uM for HOXA9. In addition the data showed that cells die from apoptosis rather than necrosis, see figure 5.4.

For this assay the specific effect of HXR9 has been calculated as the difference in percentage of apoptotic cells in samples exposed to HXR9 compared to samples exposed to CXR9. Test of significance with Student's t-test shows that the specific effect is significant for THP-1 and OCI-AML2 at 10 uM and for HL60 and HOXA9 at 20 uM (figure 5.5). For OCI-AML3 the effect is not significant at either 10 or 20 uM.

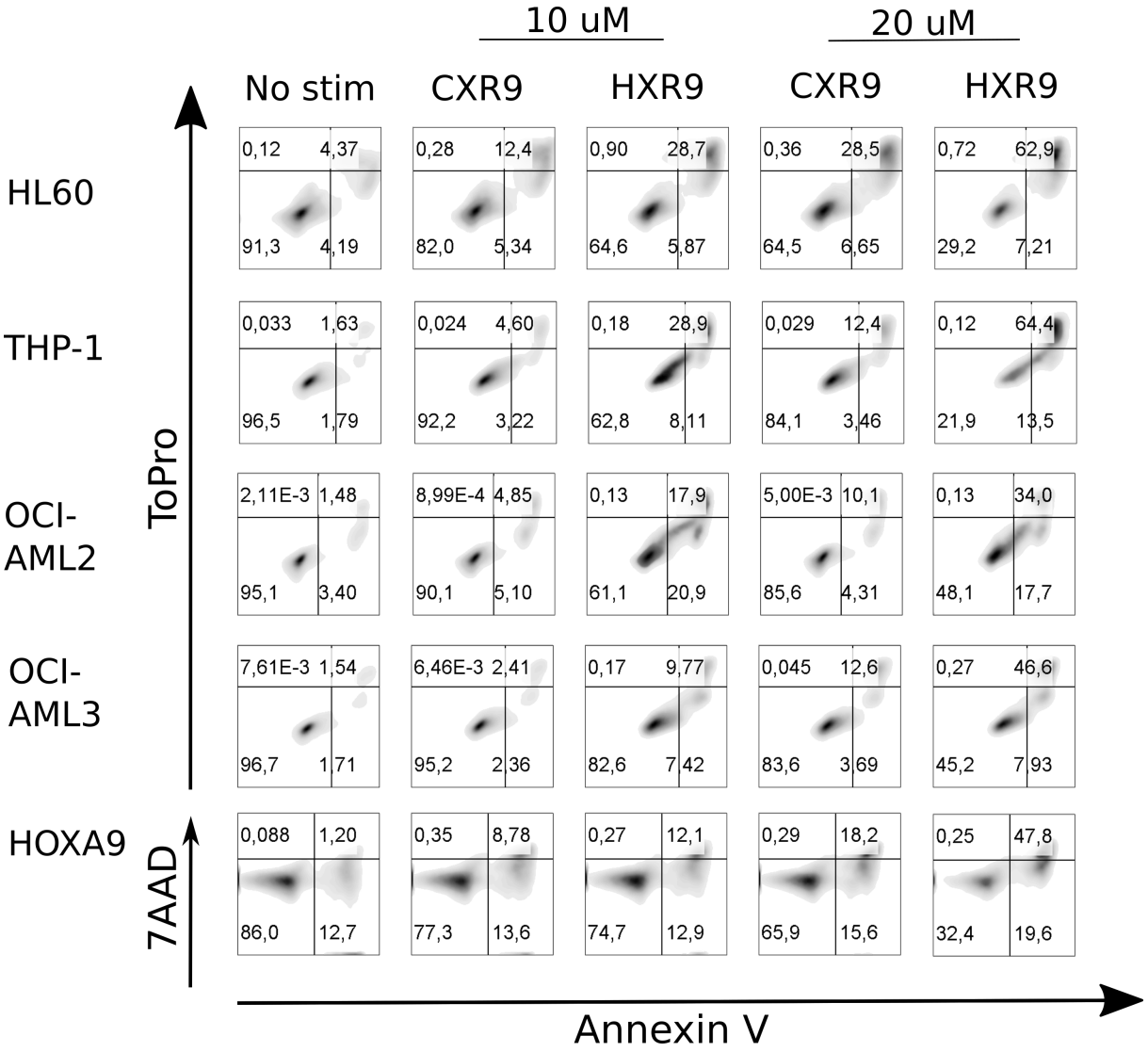


Figure 5.3: Annexin V apoptosis assay. Representative dot plots from one of three independent experiments are shown.

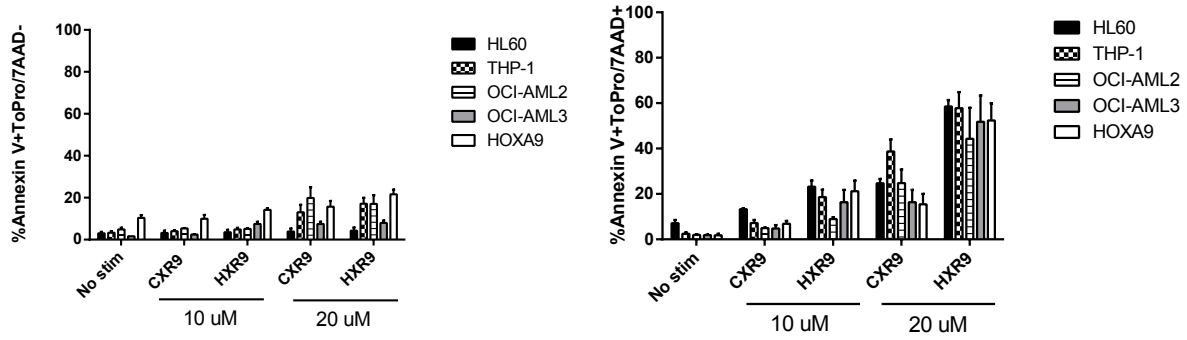


Figure 5.4: FACS annexin V apoptosis assay, with early (up) and late (down) apoptotic cells shown in separate graphs. A majority of the apoptotic cells are in the late stage. Less than 3 % of cells were annexin V-ToPro/7AAD+ (not shown) in all samples. Mean values and SEM from three separate experiments are shown.

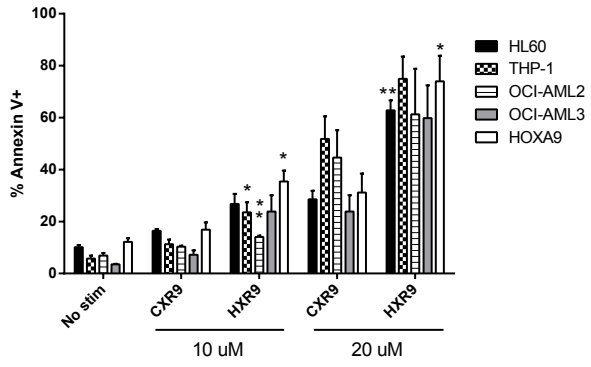


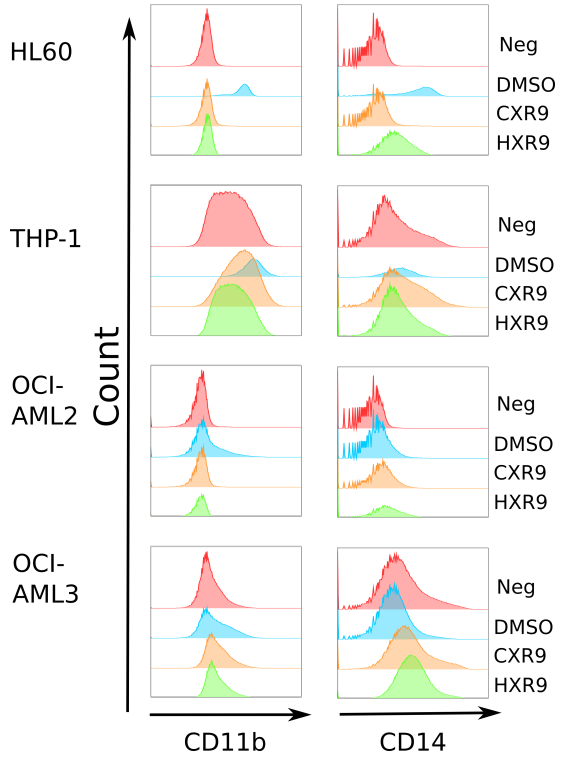
Figure 5.5: Annexin V apoptosis assay, summary of all apoptotic cells. Annexin V+ include both ToPro/7AAD+ and ToPro/7AAD- cells. One, or two * symbols denote a significant difference between HXR9 and CXR9 at the indicated concentrations, according to a student t-test with a p-value of <0.05, and 0.01 respectively. Mean values and SEM from three independent experiments are shown.

5.3 Peptide induced maturation

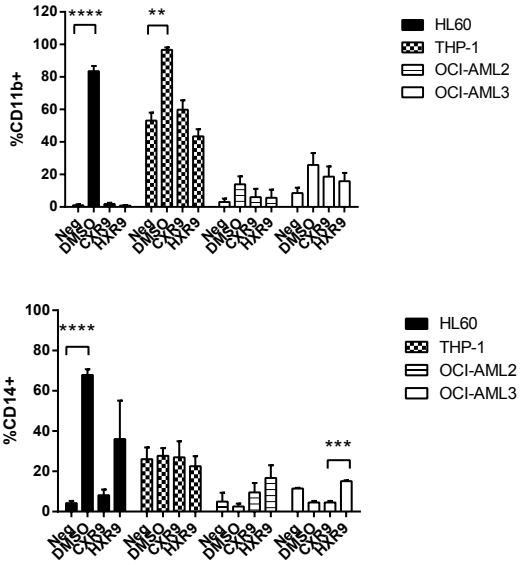
To investigate any pro-differentiating effect of HXR9, flow cytometry panels were set up, one for human and one for murine cells. Two of the human cell lines, HL60 and OCI-AML3, were also studied using a CFU-assay.

5.3.1 Human cell lines

Results from the flow cytometry panel showed that there is a trend towards increased expression of CD11b in OCI-AML3 after stimulation with HXR9 and CXR9 (figure 5.6b). HXR9 significantly increases the expression of CD14 in OCI-AML3 and there seems to be a trend towards increased expression in OCI-AML2 and HL60. The results also showed that THP-1 has a higher percentage of CD11b+ and CD14+ cells than the other cell lines without stimulation and that while DMSO significantly increases expression of CD11b and CD14 in HL60 and CD11b in THP-1, there is no significant increase in expression of either CD11b or CD14 in OCI-AML2 or OCI-AML3 after stimulation with DMSO. Representative histograms from one of three independent experiments are shown in figure 5.6a.



(a) Representative histograms from one of three independent experiments.

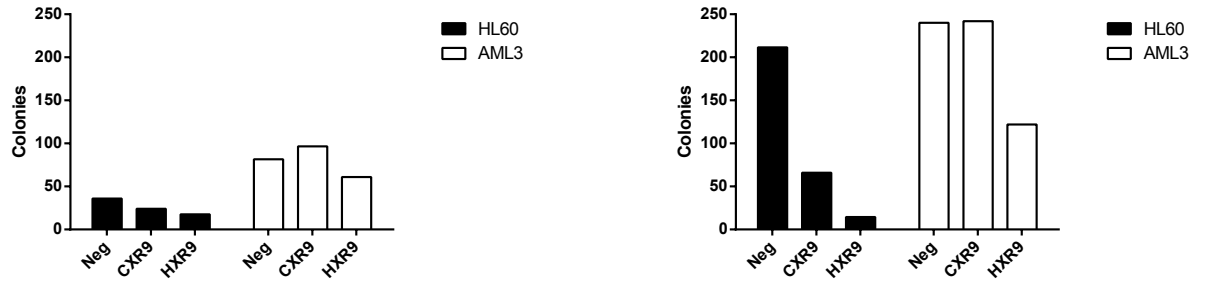


(b) Percentage of cells that are positive for the myeloid markers CD11b and CD14. One, two, three, or four * symbols denote a significant difference, according to a Student's t-test with a p-value of <0.05, 0.01, 0.005, and 0.001 respectively. Mean and SEM from three independent experiments are shown.

Figure 5.6: Maturation assay, human cell lines.

The CFU-assay showed that both HL60 and OCI-AML3 were able to form colonies. The HL60 colonies had the appearance of CFU-G, being small and dense, whereas the OCI-AML3 colonies were numerous but sparse, with the appearance of CFU-M or CFU-GM. During the first week HL60 formed less than 50 colonies per plate, but the second week it formed as many colonies as OCI-AML3 in the untreated sample. For HL60 both CXR9 and HXR9 seemed to inhibit the formation of colonies, although the effect of CXR9 was weaker (see figure 5.7). For OCI-AML3 HXR9, but not CXR9, seemed to inhibit the formation of colonies (figure 5.7). No statistical analysis could be performed as there were too few samples.

The experiment was canceled after the second week as results from the other experiments indicated that the effect of HXR9 was less specific than previously thought.



(a) First plating (b) Second plating

Figure 5.7: CFU assay. Colony count 6 days (8 days for the 1st plating) after plating of 500 cells on a small petri dish. The arithmetic mean from duplicate samples is shown.

5.3.2 Murine cells

Only two replicates of the flow cytometry maturation assay were performed on the HOXA9 liquid culture. Due to methodological difficulties the two experiments were performed nearly one month apart (2015-03-25 and 2015-04-24). The results showed a decrease in expression of the markers examined in all samples, regardless of stimulation and the results were deemed non-comparable, see figure 5.8. Thus, no synthesis graphs are shown.

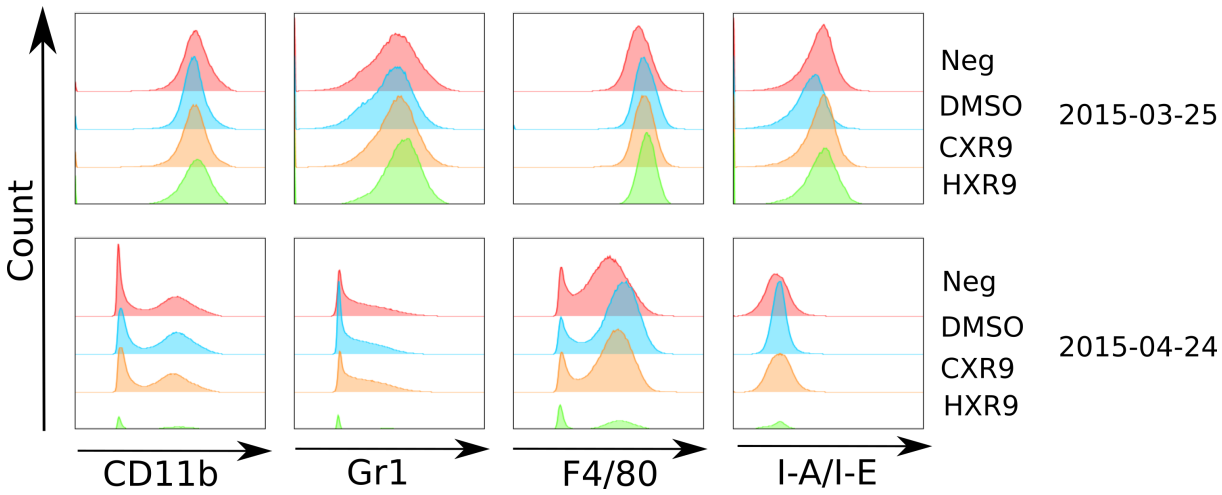


Figure 5.8: Maturation assay, HOXA9 liquid culture. Histograms from two independent experiments performed one month apart, 2015-03-25 and 2015-04-24, show different patterns of expression.

6 Discussion

6.1 Gene expression analysis

The Western blot confirmed the expression of wild type NPM in all cell lines and the presence of mutated NPM exclusively in OCI-AML3, in accordance with the distributor DSMZ[46] and Quantmeier et al [47]. The HOXA9 expression is high in positive control cell line THP-1, in accordance with previous studies [17, 28] as well as in OCI-AML2, OCI-AML3, also in accordance with previous reports [33, 22]. The liquid culture HOXA9 has a very high expression of HOXA9, which is consistent with transfection of the human HOXA9 gene. HL60 show only a low/moderate expression of HOXA9.

These results confirm that HL60 is suitable as a negative control cell line for this study as it does not carry a mutation in the NPM1 gene and has a low expression of HOXA9. Furthermore THP-1 is suitable as a positive control, with no mutation in NPM1 but a high expression of HOXA9. It is also confirmed that OCI-AML3 carries a mutation in NPM1, and can thus be used as an *in vitro* model of NPMc+ AML.

6.2 The cytotoxic effect of HXR9

The Alamar Blue cytotoxic assay showed a reduced viability of AML cells after exposure to HXR9 in a dose-dependent manner (figure 5.2). However, it is not possible to distinguish if the reduced viability is due to a reduced proliferation or an actual cytotoxic effect in this assay. The annexin V apoptosis assay showed that cells exposed to HXR9 die by apoptosis (figure 5.4), which indicates that HXR9 is cytotoxic to AML cells. According to the Alamar Blue assay, THP-1 and OCI-AML2 seem to be affected at lower concentrations, compared to HL60, OCI-AML3 and HOXA9 (figure 5.2), but the results are not conclusive. No such trend is seen in the annexin V apoptosis assay.

CXR9, intended to serve as a negative control, was also shown to cytotoxic to all the cell lines examined, although the effect was weaker than that of HXR9. These results stand in contradiction to previous studies, including Li et al [17], who have found CXR9 to work as

intended with no impact on cell viability. A possible explanation of the effect of CXR9 is its structural similarity with HXR9. Substitution of just one amino acid, even though it disrupts the hexapeptide believed to be of great importance to it's function [16, 36], may not be enough to render CXR9 completely inactive, even though mutation analysis have shown that the particular amino acid substituted in CXR9 is of vital importance to the YPWM motif [48]. CXR9 may still be able to interfere with the interaction between HOX and PBX, albeit with a weaker effect than that of HXR9. In addition, Shanmugam et al have shown that the amino acids flanking the YPWM motif contribute to the interaction between HOX proteins and PBX [48]. The flanking residues of CXR9 and HXR9 are identical and might thus contribute to the binding capacity of CXR9.

In summary,we here show that HXR9 seems to be cytotoxic to AML cells but the effect is less specific than expected as the negative control CXR9 also exhibit significant cytotoxic effects.

6.3 Pro-differentiating effect of HXR9

6.3.1 Human cell lines

Results from the maturation panel suggest an increased maturation in samples exposed to HXR9. The ratio of cells expressing the myeloid marker CD14 was increased and in OCI-AML3 the increase is significant compared to CXR9 (figure 5.6b). The effect on THP-1 was very weak, but it could be due to a lower peptide concentration of 5 uM instead of 10 uM. A lower peptide concentration was chosen for this cell line due to their increased sensitivity to the peptide and a higher concentration resulted in a reduced cell viability and trouble maintaining exposure for seven days. The effect of HXR9 on HL60 is unclear. There was a trend towards a rather dramatic increase in CD14+ population, but the measured variability was too high to obtain any statistical significance. The study may be improved and better illustrate any pro-maturating effect of HXR9 by testing an increased exposure time and possibly a higher peptide concentration.

DMSO was intended as a positive control for maturation. A first test run (data not shown) showed a low cell viability in OCI-AML2 and OCI-AML3 after exposure to 1% DMSO, which is the reason for lowering the concentration to 0.5%. This concentration, however, seems to be too low to induce maturation and thus not ideal as a positive control. Should these experiments be performed again, it would be preferable to use a higher concentration of DMSO even if it would result in a reduced cell viability.

The results from the CFU experiment are inconclusive. There is a trend towards fewer colonies in the samples treated with HXR9 (see figure 5.7), but it is not possible to distinguish if this is due to the acute cytotoxic effect, probably resulting in a reduced number of cells surviving the first day, or due to loss of proliferative capacity. If more replatings were performed and if they would have shown a continuous reduction of colonies in the samples treated with HXR9 but not in the others, this would indicate that HXR9 has pro-differentiating, anti-proliferative effect on the leukemic cells.

For OCI-AML3 there was no difference between samples exposed to CXR9 and negative controls, but there were lesser colonies in the samples exposed to HXR9. For HL60 there was a trend towards fewer colonies with CXR9 as well as HXR9, in accordance with cytotoxicity and apoptosis assays. However, too few samples were available to perform a test of significance.

Interestingly, both OCI-AML3 and HL60 formed more colonies the second week. If it was a true increase and not the result of a methodological error, it might be due to a selection of cells with high proliferative capacity.

6.3.2 Murine cells

The results from the maturation assay on the liquid culture HOXA9 showed marked decrease in expression of all the four maturation markers examined in the second experiment compared to the first one, performed approximately one month apart (see figure 5.8). In addition, there was a decrease in GFP-positve cells in the second experiment compared to the first, 60% compared to 45%. A possible interpretation is that the cells have begun to lose the HOXA9-GFP construct and possibly acquired additional mutations resulting in less mature, more leukemia-like cells.

They may also have downregulated the expression of GFP but that would not explain the decrease of myeloid markers. According to Thorsteinsdottir et al.[10], it takes seven months for mice injected with HOXA9 overexpressing cells to develop AML, which indicates that overexpression of HOXA9 alone is not enough to cause AML, but requires the acquisition of additional mutations.

A delay of one month between experiments is far from optimal, especially when working with a liquid culture that is more similar to primary bone marrow cells than the established cell lines.

6.4 The efficiency of HXR9 in relation to the expression level of HOXA9

The working theory of this project was that HXR9 should induce apoptosis and possibly maturation in AML cells with a high expression of HOXA9, due to the inhibition of the interaction between HOXA9 and PBX3. However, the results from all experiments presented in this study, showed that both HXR9 and CXR9 are cytotoxic to and possibly pro-maturating for HL60, which has a very low expression of HOXA9, according to the Western blot. Inhibition of HOXA9 should have no or at least very little effect on these cells.

One of many possible explanations for the deviant results are that an unknown event occurred affecting the peptides and rendering them unspecific. However, it seems improbable that such an alteration would enhance the effect of CXR9, as one would instead expect a weakened effect. Another explanation may be that something added at the same time as the peptides was harmful to the cells. However, the peptides were dissolved in distilled water and diluted in the same medium that the cells were cultured in and nothing else was intentionally added. As the peptides were diluted at least 1:5,000 before coming in contact with the cells, any osmotic event would too weak to be explain the phenomenon.

The most probable explanation, however, concerns the proposed mechanism of action of the peptide. Opinions seems to differ about which HOX proteins contain a YPWM motif, the sequence mimicked by HXR9. According to Morgan et al [35], the YPWM sequence is found in HOX proteins of paralog groups **1–9**, with references to others: Shanmugam et al [48],

Chang et al [36], and Shen et al [49]. However, these authors all agree that YPWM is only found in paralog groups 1–**8**. According to Shen et al HOX proteins of paralog groups 9–13 do not have a YPWM motif, but HOX proteins of groups 9–10 still bind to PBX1 [49]. If the hexapeptide is not found in HOXA9, it would not be surprising that the effect of HXR9 is not dependent on expression of HOXA9. Also, as the hexapeptide is present on all HOX proteins of paralog groups 1–8/9, inhibition of the interaction with cofactor PBX3 would affect all these other HOX proteins as well, possibly providing enough noise to erase any sign of correlation between efficacy of HXR9 and expression level of HOXA9.

None of this explains the discrepancy between the results of Li et al and the results of this study [17]. They tested the exact same peptides on the exact same cell lines (HL60 and THP-1) but found that neither HXR9 nor CXR9 had any effect on HL60, and only HXR9 had any effect on THP-1, in accordance with their expression of HOXA proteins. However there are a several circumstances that can complicate a comparison:

1. The use of fetal calf/bovine serum in the culture medium. Serum is ill-defined and there is a high variability between batches [50].

2. In their paper, Li et al. do not state exactly how the peptides were diluted and stored or what cell concentrations they used [17].

3. Li et al. used another cell viability assay in their study [17].

6.5 Improvements and further investigations

Several improvements to the experiments performed are possible, such as to avoid long periods of time between experiments and minor adjustments to concentrations of peptides. Also, to exclude any disturbance caused by the fetal calf serum one could use a serum-free medium. This would also eliminate problems with variability between batches of serum.

Concerning the lack of specificity of HXR9, one important aspect to consider is that HXR9 and the YPWM-motif do not seem to affect HOXA9 alone, but also many other HOX proteins that collaborate with PBX3. Therefore, it would be of interest to explore the expression of other HOX proteins and their relation to the efficacy of HXR9. It would also be interesting to

examine whether changes to the amino acid sequence of HXR9 or CXR9 would have an impact. For example, one could substitute both the tyrosine (Y) and the tryptophan (W) residues of the YPWM-motif in CXR9, as these seem to be the most important ones for its binding to PBX3 [48], and clarify if that would render it less efficient and more suitable as a negative control.

Once specificity is obtained, the next step in the investigation of HXR9 as a possible treatment would be to perform tests *in vivo*. A mouse xenograft model using OCI-AML3 cells is under esablishment.

6.6 Conclusions

HXR9 exerts a cytotoxic and possibly pro-maturating effects on AML cells, but the effect may be unspecific and independent of HOXA9 expression level. HOX-inhibition could still be a potential future treatment option for AML but in the case of HXR9 more extensive studies need to be performed concerning its mechanism of action, and a higher specificity needs to be achieved.

7 Populärvetenskaplig sammanfattning

HOX-inhibitorn HXR9:s verkan i akut myeloisk leukemi

I det här examensarbetet undersöktes huruvida substansen HXR9 skulle kunna vara en kandidat för behandling av akut myeloisk leukemi.

Akut myeloisk leukemi (AML) är den vanligaste typen av akut leukemi — blodcancer — i Sverige och som namnet antyder är förloppet hastigt. Dödligheten är hög, endast ca 20–30% av patienterna lever 5 år efter diagnos. Prognosen är dock tydligt bättre för yngre patienter (<60 år).

Liksom alla typer av cancer uppstår AML då en frisk cell muterar, vilket innebär att det uppstår förändringar i DNA. Mutationer som leder till cancer kännetecknas av att de gör att celler får lättare att växa och dela sig och att de inte längre samarbetar med resten av kroppen.

Det är mycket intressant att veta exakt vilka mutationer som finns i cancerceller eftersom mutationerna är tänkbara mål för läkemedelsbehandling. Nya cancerläkemedel som verkavia sådana mutationer kan både vara mycket effektiva, eftersom det är just mutationerna som gör cancercellerna så aggressiva, och dessutom kan de ge mindre biverkningar än t.ex. cellgifter, eftersom ett dessa nya läkemedlel bara påverkar cancercellerna och inte de friska cellerna. HXR9 är en substans som är designad att verka via mutationer som finns i just AML-celler.

I det här arbetet undersöktes vilken effekt substansen HXR9 har på AML-celler. Alla experiment har skett på laboratorium och de undersökta cellerna kommer från etablerade cellinjer som kan odlas där. För att kunna besvara frågeställningarna om HXR9:s effekt på AML-celler har flera undersökningar gjorts:

- Kontroller för att bekräfta att cellerna har de egenskaper som förväntades.
- Celler utsattes för HXR9 och sedan undersöktes hur stor andel av cellerna som dött vid olika koncentrationer samt huruvida cellerna dött kontrollerat eller okontrollerat. Okontrollerad celldöd är mer skadligt för omkringliggande celler och vävnad.
- Celler utsattes för låga koncentrationer av HXR9 under en längre tid och sedan undersök-

tes cellerna för att se om de utvecklats till mer mogna celler. Detta är intressant då mogna celler är mindre aggressiva och lättare att komma åt med redan etablerad leukemibehandling.

Resultaten från experimenten visar att HXR9 är skadligt för AML-celler och gör att de dör kontrollerat. Vid experimenten användes en annan substans, CXR9, som negativ kontroll. Denna är mycket lik HXR9 men en liten strukturell skillnad gör att den borde vara verkningslös. Experimenten visar dock att CXR9 också har förmåga att döda AML-celler, även om effekten är svagare än för HXR9. Försöken som inriktades på att se om HXR9 kunde få AML-celler att mogna visar att det finns en sådan trend, men den är svag och det går inte att utesluta att den beror på slumpen.

Sammanfattningsvis visar arbetet att HXR9 kan orsaka celldöd och eventuellt ökad cellmognad vid AML, men effekten är inte så specifik som förväntat. Behandlingar som inriktas på specifika mutationer är dock fortfarande högintressanta vid såväl AML som andra typer av cancer.

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A Alamar Blue Assay

A.1 Equations

Equations provided by the data sheet for the Alamar Blue Cell Viability Reagent (Life Technologies).

$$\frac{(\varepsilon_{OX})\lambda_2 A\lambda_1 - (\varepsilon_{OX})\lambda_1}{A\lambda_2(\varepsilon_{RED})\lambda_1 A'\lambda_2 - (\varepsilon_{RED})\lambda_2 A'\lambda_1} \tag{A.1}$$

$$\frac{(\varepsilon_{OX})\lambda_2(A\lambda_1 - A'\lambda_1) - (\varepsilon_{OX})\lambda_1(A\lambda_2 - A'\lambda_2)}{(\varepsilon_{OX})\lambda_2(A^o\lambda_1 - A'\lambda_1) - (\varepsilon_{OX})\lambda_1(A^o\lambda_2 - A'\lambda_2)} \tag{A.2}$$

Table 4: Definition of variables for Alamar Blue equations.

VARIABLE	DEFINITION
λ_1	570 nm
λ_2	600 nm
$\varepsilon_{(OX)}\lambda_1$	Molar extinction coefficient of Alamar Blue oxidized form (blue) for wave-length $\lambda_1 \rightarrow 80596$
$\varepsilon_{(OX)}\lambda_2$	Molar extinction coefficient of Alamar Blue oxidized form (blue) for wave-length $\lambda_2 \rightarrow 117216$
$\varepsilon_{(RED)}\lambda_1$	Molar extinction coefficient of Alamar Blue reduced form (red) for wave-length $\lambda_1 \rightarrow 155677$
$\varepsilon_{(RED)}\lambda_2$	Molar extinction coefficient of Alamar Blue reduced form (red) for wave-length $\lambda_2 \rightarrow 14652$
A	Absorbance of test well
A'	Absorbance of negative control well. This well should contain media and Alamar Blue reagent, but no cells.
A^o	Absorbance of positive growth control well. This well should contain cells, but no cytotoxic agent.

A.2 Supplemental figure

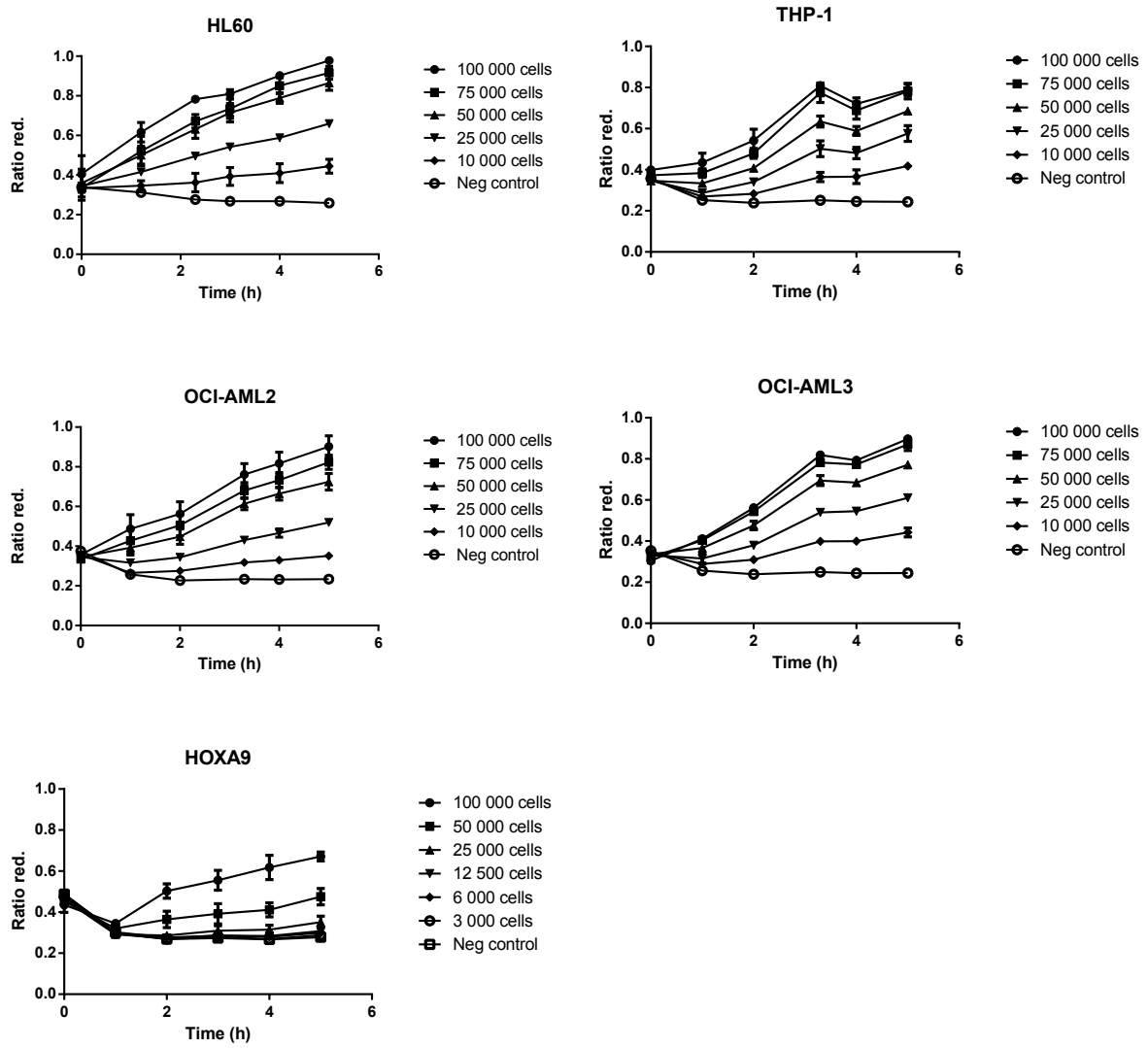

Figure A.1: Test runs for optimization. The incubation time for the Alamar Blue reagent was set to 4 hours for all cell lines and cell counts to 100,000 for HOXA9, 75,000 for HL60, OCI-AML2 and OCI-AML3 and 50,000 for THP-1. Mean values and $\pm$ 2SD from one experiment are shown.