Differentiation Therapy of Acute Myeloid Leukemia

- Deciphering the mechanism of action of candidate molecule H4.

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Potential Future Differentiation Therapy For Acute Myeloid Leukemia

Did you know that a small percentage of acute myeloid leukemia cases have a far better survival rate than the rest? This is achieved by inducing maturation of the blood cancer cells that are trapped in an immature state (a process called differentiation therapy) and thereby restoring normal blood cell production. However, only 10% of AML patients can be treated in this way. Here we investigate by which mechanism our candidate H4 induces differentiation and our results suggest that H4 increases sensitivity of the leukemic cells to normal blood cell maturation signal IL-3.

Acute myeloid leukemia (AML) is the most common form of acute leukemia in adults and is characterized by the accumulation of immature blood cells which "clog up" the bone marrow and prevent the normal production of blood cells. Patients are generally treated with chemotherapy, which targets all dividing cells in the body and often results in severe side-effects. The treatment effectively kills a majority of the leukemic cells, but is very ineffective when it comes to eradicating the so-called leukemic stem cells (LSCs) which are believed to be driving the disease. This results in a high relapse-frequency and only 1 in 4 patients are still alive five years after receiving the diagnosis.

However, AML patients with the subtype acute promyelocytic leukemia (APL) which represents around 10% of AML cases can be successfully treated with all-trans retinoic acid (ATRA) resulting in a survival rate of approximately 80%2. Due to a mutation characteristic for APL, treatment with ATRA forces the immature blood cells to mature, which unclogs the bone marrow and allows for normal blood cell production to resume. By forcing the leukemic cells to mature instead of simply killing them off, the LSCs are also forced to mature, thereby losing their ability to cause a relapse. This concept of unlocking the blocked maturation is referred to as "differentiation therapy" and is especially appealing as it avoids the side effects of cytotoxic chemotherapy by maturing cells instead of killing them. More importantly, it also increases the chance of complete remission.

There is therefore great interest in finding similar maturation-enforcing differentiation therapies for the remaining 90% of AML cases. We have identified a candidate molecule (code name "H4") that successfully increases cell maturation in AML cell lines and post-relapse patient samples based on what surface markers (tags which indicate group affiliation) the cells express. How H4 interacts with the cells is not currently known, but has been hypothesized to be connected to increased kinase signaling. I have examined which signaling pathways in the cell are involved in the interaction. Results point towards treatment with H4 increasing the cells’ sensitivity to IL-3, a signaling substance which is a signal for maturation in normal blood cell development. This could be one factor in how H4 forces the leukemic cells to mature, and that knowledge can be used to design a future differentiation therapy for AML and improve patient survival rates.

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Summary

Acute myeloid leukemia (AML) is characterized by a differentiation block in the myeloid lineages of the hematopoiesis. After the success of the all-trans retinoic acid (ATRA) differentiation therapy which overcomes this differentiation block for AML subtype acute promyelocytic leukemia, attempts have been made to find similar therapies for the remaining subtypes. Differentiation therapy candidate H4 (derived from a library of small natural products) has been shown to induce phenotypic differentiation in both an AML cell line and a primary AML sample. In this study, we show that treatment with H4 increases the sensitivity to IL3, which is a differentiation-promoting cytokine in normal hematopoiesis, by upregulating expression of its receptor. This knowledge can be used to design future differentiation therapies for AML.

Introduction

Hematopoiesis is the constantly on-going process by which blood cells are generated in the body. This takes place in the bone marrow where hematopoietic stem cells (HSCs) proliferate and differentiate into more specialized progenitors, and then further into the various types of mature cells found in the blood. The HSCs have the option of self-renewal (where both daughter cells are identical to the mother cell), differentiation or apoptosis upon cell division, and will chose their fate based on a balance between an intrinsic program and signals received from their surroundings. Hematological malignancies such as leukemia arise when this cell fate program is disturbed. (Doulatov et al., 2012)

Acute myeloid leukemia (AML) is the most common form of acute leukemia in adults and results in the accumulation of undifferentiated myeloid cells, which crowd the bone marrow, leading to an impaired hematopoiesis and symptoms such as anemia and thrombocytopenia. The characteristic differentiation block can occur at various stages of the myeloid differentiation, leading to different subtypes of AML depending on which progenitors are accumulating. A combination of pro-proliferative and anti-differentiation events are necessary for the rise of AML, for example chromosomal translocations. (De Kouchkovsky and Abdul-Hay, 2016)
AML is a heterogenous disease, both biologically and clinically, and is divided into six major groups based on genetics, morphology, immunophenotype and clinical presentation. Important factors in deciding which type of therapy to use in each individual case are prognostic factors such as risk of treatment resistance and treatment-related mortality. This assessment is largely based on cytogenetic changes and molecular abnormalities, which are divided into "favorable", "intermediate" and "adverse". For example, AML caused by therapy or by a prior hematological malignancy are associated with a poorer prognosis. (De Kouchkovsky and Abdul-Hay, 2016)

Conventional therapy for AML involves various regiments of chemotherapy based on cytotoxic drugs which target cycling cells and therefore kills only proliferative cells. There is however a population of leukemic cells referred to as leukemic stem cells (LSCs) which are believed to be at the top of the AML hierarchy in the same way the healthy HSCs are in the normal hematopoiesis. LSCs also share properties with healthy HSCs such as quiescence and the ability to efflux drugs (Pabst et al., 2016; Yanagisawa et al., 2016). This allows them to escape the treatment and re-initiate the leukemia, consequently causing a post-treatment relapse, which poses a therapeutical challenge as treatment with cytotoxic drugs is no longer effective (De Kouchkovsky and Abdul-Hay, 2016).

An alternative treatment strategy is to target or bypass the differentiation block and restore myeloid differentiation of the accumulated cancer cells. This is referred to as differentiation therapy, currently used in the clinic for treatment of AML subtype acute promyelocytic leukemia (APL) using all-trans retinoic acid (ATRA) in addition to conventional chemotherapy (Marchwicka et al., 2014). A characteristic translocation in APL leads to an insensitivity to physiological levels of the retinoic acid differentiation signal due to the fusion protein PML/RARα blocking the retinoic acid receptor from binding to the promoter region. The differentiation block is overcome by giving the patient large doses of ATRA, which helps restore normal retinoic acid signaling. After the success of the ATRA differentiation therapy in improving the five year survival rate (>80% for APL compared to the general 27% for AML), there is a desire to find similar therapeutic options for the remaining 90% of AML cases (Coombs et al., 2015; NIH, 2017).
The laboratory of Mattias Magnusson has previously reported on the ability of Indirubin to differentiate cytogenetically diverse AML samples in a co-culture system with OP9M2 stromal cells (Baudet et al., 2016). The OP9M2 co-culture system was originally described as supporting human HSC function in vitro (Magnusson et al., 2013) due to their mimicry of the haematopoietic niche in the bone marrow, and this can be utilized for the study of LSCs as they are also supported by this niche and prevented from undergoing spontaneous differentiation in vitro. The evaluation of differentiation requires the study of multiple parameters including changes in immunophenotype (CD11b and CD15 are common myeloid differentiation markers), morphology (cell size and granularity), cell count, cell cycle (ratio of cycling to non-cycling cells), apoptosis (cell death), and functionality of the differentiated cells (e.g. phagocytosis for mature macrophages).

Using the same screening platform, candidates for differentiation therapy of genetically diverse AML were found in a screen of a library of small molecules derived from natural sources on AML primary samples and cell lines (unpublished data). Natural products were used due to their great structural diversity, which allowed for novel findings.

Preliminary results showed the molecule H4 found in the screen to be capable of increasing the expression of myeloid differentiation markers such as CD11b and CD15, thereby indicating a potential use for differentiation therapy. In an attempt to decipher the mechanism of action and understand which pathways are involved in the effect of H4, a microarray analysis of an H4-treated AML primary sample was conducted. Results from this indicate altered kinase signaling. In addition, the molecular structure (although undisclosed) supports this theory as it resembles known kinase modulators. Therefore, H4 was combined with known kinase inhibitors (KIs) using AML cell line U937. Notably, 8 candidates, together targeting five different pathways, were selected for further testing based on their ability to potentiate the effect of H4.

In this study, I have further characterized the effect of H4 on differentiation, apoptosis and cell cycle in both primary sample and cell lines. I was also able to confirm the potentiating effects of the KIs on the differentiating effect of H4 on U937 cells. However, while most of those compounds similarly affect the differentiation potential of H4 on a primary AML sample, we found that c-Raf
inhibition inhibits the effect of H4, in particular for the upregulation of CD123 (the α-subunit of the IL3-receptor).

IL3 is a cytokine involved in normal hematopoiesis where it induces growth as well as differentiation (Reddy et al., 2000). The signal from the IL3-receptor is mediated through various pathways, including the JAK/STAT-pathway and the ERK-pathway where c-Raf is located (Reddy et al., 2000). As H4 increases the expression of the IL3-receptor, the resulting increased sensitivity to IL3 could be one factor in how H4 induces differentiation. H4 was confirmed to increase sensitivity to IL3 using a model of cord blood derived HSCs.

Results

The natural product H4 induces phenotypic differentiation of human AML primary samples and cell lines.

The natural product H4 was able to differentiate several AML samples during the screening process (data not shown). In order to validate the effect of H4 on AML cells, we first treated either the AML cell lines U937 and MonoMac6 (MM6, later excluded from the project as it was not growing at the expected rate) or a primary AML sample (A4, a chemotherapy-resilient post-relapse sample with a complex karyotype of which we have a substantial supply) with 2.5 and 5 µM of H4. After 4 days of treatment, FACS analysis of surface markers expression demonstrated a 4-fold increase of the expression level of CD11b at 5µM H4, as demonstrated by FACS plots and quantification of mean fluorescence intensity (MFI) (figure 1A). A smaller (less than 2-fold) increase was observed when cells were treated for 2 days or with a 2.5µM dose, indicating that a treatment of 5µM H4 for 4 days is required for a strong response. The expression level of CD15, although slightly increased compared to the non-treated control, was not further altered by dose or duration of H4-treatment. Additionally, cells were stained with May-Grünwald and Giemsa (MGG) solutions to study morphological changes (figure 1B). This was done after four days of treatment and showed very little difference between non-treated and 2.5µM H4. However, at 5µM H4, cells increased in size and granularity which can be interpreted as a sign of myeloid differentiation,
confirming the changes in phenotypes. In addition, cells with morphological similarities to mature macrophages in terms of size and cytoplasmic granules were generated.

Parallel, when treated with 5µM of H4 for 4 days, while the expression profile of CD11b remained unchanged (figure 1C) for the primary sample A4, a 3-fold increase of the percentage of CD15+ cells (figure 1D) was demonstrated. Furthermore, microarray analysis had shown that two other well characterized myeloid markers, CD123 (the α-subunit of the IL3 receptor, (Borriello, Longo et al. 2015)) and CD64 (specifically expressed on normal macrophages, (Yong, Kim et al. 2017)) are
upregulated in 16 hours-treated A4 cells (data not shown). In fact, after 4 days of treatment with 5µM H4, the percentage of CD64+ cells reached more than 50% compared to approximately 10% in control-treated cells (figure 1E). The most notable effect was the induction of CD123 expression in 11% of the cells while it remained undetected in control cells (figure 1F).

Finally, while unchanged when A4 was treated by H4, the cell count in U937 decreased at almost 50% of the non-treated control (figure 1G-H).

Natural product H4 increases apoptosis in human AML cell line, but not in primary sample.

To address the reason behind the decrease in cell number following H4 treatment, we treated either the AML cell line U937 or a primary sample A4 with 2.5 and 5 µM of H4 and assessed the level of apoptosis by FACS analysis of annexin V (anxV) after 2 and 4 days of treatment. Unaffected by the 2.5µM dose, the percentage of AnxV+ cells in U937 was upregulated by a 4-fold factor after 2 days of treatment by 5µM and remained stable at day 4 (figure 2A). In addition, a significant majority of the AnxV+ cells could be found in the CD15high population with 10% anxV+ cells in CD15hi vs 5% in CD15lo (figure 2B). Alternatively, a 30% decrease could be observed when A4 primary cells were treated for 4 days with 5µM of H4 independently of the subpopulation (figure 2C).

Natural product H4 marginally increases cell division in human AML primary sample.

Alternatively to increased apoptosis, the decrease in cell number observed in U937 could be the results of changes in proliferation status. To assess the effect of H4 on cell cycle, we treated the AML cell line U937 with 2.5 and 5 µM of H4 for 4 days prior to DNA staining and FACS analysis. Several staining protocols were tested on fixated and permeabilized U937 cells (figure 3A). Using 7AAD following RNA digestion with RNaseA lead to a split G1 peak and the lack of a G2+M peak. Using Nuclear-ID® Red DNA Stain failed to generate a recognizable curve for analysis. Finally an attempt was made using propidium iodide (PI) which proved successful and this staining was

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therefore used for the subsequent cell cycle experiments. No significant differences could be detected in the distribution within the different cell cycle stages (G1, S and G2+M) in U937 following treatment with H4 (figure 3B).

Although the cell count of primary sample A4 was not affected by H4 treatment, changes in cell cycle status are linked to differentiation and quiescence of LSC. Therefore, the A4 primary cells were stained with proliferation marker Ki67 in addition to DNA content analysis by PI staining. After 4 days of treatment by a 5µM dose of H4, a slight decrease of the percentage of cells in the non-cycling G0-phase was observed when compared to control treated cells (figure 3C).
Inhibition of c-Raf decreases the effect of natural product H4 on CD123 expression in human AML primary sample while inhibition along other pathways increase it further.
Eight candidates from a previously conducted screen (data unpublished) of known kinase inhibitors (KIs) for a potentiating effect on H4 were validated on AML cell line U937 (figure 4A) and primary sample A4 (figure 4B). These eight candidates were PP1 and PP2 (targeting members of the Src family), ML7 and ML9 (targeting MLCK), RG14260 and Tyrphostin 23 (targeting EGFRK), ZM336372 (targeting c-Raf) and Rottlerin.

The first iteration of this validation experiment was excluded due to precipitate formation in some of the wells, despite the fact that the listed solubilities for each of the inhibitors in DMSO had been followed. The KI solutions were further diluted in DMSO prior to repeating the screen and this time no crystals were seen.

We treated either the AML cell line U937 or the primary sample A4 with 2.5 and 5 µM of H4 in combination with the individual KIs at different concentrations. Vehicle treated controls were used for both H4 and KIs to allow for assessment of the effect of the KI alone. At the FACS analysis after 4 days of treatment, results confirmed the additive potentiating effect of Src-inhibition in the cell line U937 (figure 4A) as shown by the 25% increase of CD11b mean fluorescence intensity (MFI), but not in the primary sample (figure 4B). In contrast, MLCK-inhibition did not have a potentiating effect in U937, but a synergistic one was observed in the primary sample as a 2-fold increase in the CD123+ % of cells. EGFRK-inhibition, however, had a potentiating effect on H4 in both cell types. This effect was additive in the cell line with a 25% increase and synergistic in the primary sample where the EGFRK-inhibitors had little to no effect alone and increased the effect of H4 2-fold. Rottlerin was shown to have an additive effect leading to an 30% and 2-fold increase of the expression of CD15 in U937 and A4 respectively, and was in U937 observed to homogenize the otherwise divided cell populations. Notably, the inhibition of c-Raf decreases the effect of H4 on CD123-upregulation by 50% in the primary sample, but did not affect the cell line.

**Natural product H4 increases sensitivity to IL3 in CD34+ progenitor cells**

The observed upregulation of CD123 could indicate an increased sensitivity to IL3 following treatment with H4. To test whether H4 increases sensitivity to IL3, we performed an IL-3 titration
assay with or without 5µM H4 on cord blood derived HSPC cells (CD34+) as they had previously shown sensitivity to H4 treatment (data not shown). After 4 days of treatment, the expression profile of CD34 and CD90, markers allowing to track stem and progenitor activity in vitro, was established by FACS (figure 5A).

Figure 5: Natural product H4 increases sensitivity to IL3 in CD34+ progenitor cells. A) The effect of H4 and IL3 concentration on the expression of CD90 and CD34 was assessed by FACS at day 4 of treatment of cord blood-derived CD34+ cells by 5 µM H4 with different concentrations of IL3 in the culture medium. Plot shows representative profile for a vehicle-treated (NT) sample with gates for CD34+ cells, multi-potent progenitors (MPPs) and hematopoietic stem and progenitor cells (HSPCs.) B) Graph shows the quantification of the gates defined in the FACS plot at IL3 concentration 0µM as % of total cells. Data are from one experiment realized in duplicate. C) Graph shows the quantification of the gates defined in the FACS plot at different IL3-concentrations as % multi-potent progenitors (MPPs) of total cells normalized to non-IL3 treated cells. Data are from one experiment realized in duplicate.

First, treatment with H4 alone at 5µM without IL3 led to a decrease in both the CD90+ HSPCs and the CD34+ multi-potent progenitor cells (MPPs), along with a consequent increase in the more differentiated CD34+ cells (figure 5B). Second, the presence of IL3 in the culture led to a decrease in MPPs following an increase in IL3-concentration (figure 5C). Data is normalized to non-IL3 treated cells. In the presence of H4 this effect was potentiated independently of the concentration of IL3 as seen by the further decrease in MPPs despite normalization to account for the effect of H4 alone.

Discussion

H4 has been shown to induce differentiation of both AML cell line U937 and primary sample A4, but in different ways. Treatment with 5µM H4 causes an increased expression of myeloid differentiation markers CD11b and CD15 in U937 and of CD15, CD64 and CD123 in the primary
sample. Increased CD11b expression is a common way to assess myeloid differentiation in U937 as it is a marker for the maturation of myeloid precursors (Booker et al., 2015). The highest effect on CD11b in the cell line is observed after four days of treatment with 5µM H4, and therefore one possible reason for the lack of effect on this marker in the primary sample could be that the treatment is not long enough or at a high enough dosage since an effect on CD11b was observed in the primary sample in the original screen where 10µM H4 was used. The fact that the cells in the primary sample are mainly quiescent at the start of the experiment, while the cell line is actively cycling, could account for differences in differentiation stage at day 4 of treatment as cycling is required for differentiation to occur and the cell line therefore is one step ahead of the primary sample. It would therefore be of interest to study if a longer treatment with H4, with several doses administered sequentially, would cause the primary sample to reach a later stage of differentiation.

Notably, cells morphologically similar to mature macrophages in terms of size and granularity of cytoplasm could be observed following 5µM treatment. To confirm whether these are in fact mature macrophages or simply appear as such their functionality would have to be assessed, e.g. in the form of a phagocytosis assay as mature macrophages are capable of phagocytosis.

Annexin V is a surface staining used to detect the early stages of apoptosis as its target is only located on the cell surface following a phospholipid translocation occurring when the integrity of the cell membrane is compromised. The amount of apoptotic annexin V+ cells increased in the cell line upon treatment with 5µM H4, while it decreased in the primary sample. Combined with the increase in differentiation markers, this can be interpreted as the cells in U937 entering apoptosis due to terminal differentiation, while the primary cells have not yet reached sufficient differentiation. The statistically significant difference where a majority of anxV+ cells is found in the CD15hi population also supports this theory of increased terminal differentiation, as these CD15hi cells have differentiated further (based on surface markers) prior to entering apoptosis. Terminal differentiation occurs when the cells have differentiated to such an extent that they can no longer divide and differentiate further. This is an end goal of the differentiation therapy of the AML cells as
the leukemic stem cells (LSCs) will have lost their capacity for self-renewal and no longer be able to drive the disease (Miller et al., 2013).

In the cell cycle assay, while no significant difference could be detected for U937, the slight decrease in the G0 phase in the primary sample could indicate that H4 induces cell division in previously quiescent cells. However, additional biological replicates are required to assess whether this result is significant. The induction of cell division in previously quiescent cells is necessary in AML therapy in order to target these cells as both the induction of differentiation and the sensitivity to cytotoxic drugs used in chemotherapy requires the cells to be actively cycling (Saito et al., 2010). This could also be a reason for the difference in treatment response between the cell line and the primary sample, as the cells in the primary sample will mainly be quiescent at the start of the experiment while the cell line is actively cycling. In a future clinical application, this means that longer treatment with multiple doses of H4 will likely be required to target the quiescent LSCs. It could also indicate that the more differentiated and actively cycling AML blast cells will be more sensitive to H4, though this will be more difficult to assess in vitro.

The combined treatment of H4 and kinase inhibitors show that the effect of H4 can be further increased by the inhibition of selected signaling pathways. Src family kinases are involved in a wide range of cellular processes including differentiation, proliferation and migration (Espada and Martin-Perez, 2017). EGFRK or epidermal growth factor receptor kinase is mainly involved in a pathway promoting cellular proliferation (Wee and Wang, 2017). MLCK or myosin-light chain kinase is considered a regulator of cell motility, but is also involved in multiple different pathways (Khapchaev and Shirinsky, 2016). The results from validation experiments differ from the original screen, as the potentiating effect could not be reproduced for all KIs. The effect of the addition of kinase inhibitors also differs between cell line and primary sample, indicating once again that the cell types are not affected equally by the treatment both with H4 and with KIs. In addition, the potentiating effect can be synergistic in the primary sample, while additive in the cell line. The difference between how the two cell types responded to the combination of H4 and kinase inhibitors is most likely due to the difference in cell cycle status along with the cells having different
mutations present since AML is very heterogeneous (De Kouchkovsky and Abdul-Hay, 2016). This heterogeneity exists both between AML cases and within each AML case (Yanagisawa et al., 2016). Consequently the cells respond differently to therapy, with subpopulations such as LSCs having a higher chemo-resistance (Yanagisawa et al., 2016). By homogenizing traits of the heterogeneous AML cells, such as cell cycle status (quiescent or actively cycling) or surface marker expression, these traits can be targeted. For example a combination of cell cycle-induction and cytotoxic drugs could be used.

Treatment with Rottlerin (with and without H4) leads to a large increase in the expression of myeloid differentiation marker CD15 in both cell line and primary sample. In the case of cell line U937, the combination of Rottlerin and H4 results in a homogenization of the otherwise divided population. Rottlerin was originally believed to target PKC-\(\alpha\), but has recently been found to be far less specific (Soltoff 2007). As the target of Rottlerin is currently unknown, further investigation was considered outside the scope of this project.

From our phenotypic differentiation assay we know that H4 increases the expression of the IL3-receptor (CD123 is the \(\alpha\)-subunit of the IL3-receptor) in the primary sample. This effect is notably decreased by 50% following c-Raf inhibition. The fact that c-Raf inhibition does not affect H4 in the cell line could be due to the lack of IL3-supplementation in our experimental setting. In addition, the expression of CD123 was not evaluated in the cell line. The decrease in CD123 expression could also be connected to the blocked signaling along the ERK-pathway downstream of the IL3-receptor as this is where c-Raf is located (Reddy et al., 2000). No decrease in CD123 is observed for the inhibitor alone, but this could be connected to the very low baseline as the constitutive expression of CD123 in the cells is very low. c-Raf inhibition also decreases the effect of H4 in the primary sample on the expression of CD15 (data not shown) which we can speculate to indicate that part of the effect on CD15 is mediated by the ERK-pathway. In order to assess whether the decreased effect on CD123 in the combination treatment is due to the combination with H4, indicating that part of the effect of H4 is mediated by that pathway, or due to c-Raf alone it would be necessary to repeat the experiment with cells that have a higher constitutive expression of
CD123 as this would give a higher baseline. An increased expression of the IL3-receptor could indicate an increased sensitivity to IL3 and, as the cytokine is a signal for growth and differentiation in normal hematopoiesis (Reddy et al., 2000), an increased IL3-signaling could be one aspect of the mechanism of action of H4. However, at this stage of the experiments a connection between H4 and IL3-sensitivity merely correlation and a lot of additional testing will be required to support the hypothesis that H4 increases IL3 sensitivity.

Our IL3-titration experiment with CD34+ cord blood cells shows that an increased IL3 concentration leads to an increased differentiation as seen in the decrease in CD34+ HSPCs and MPPs. The CD90+ CD34+ HSPCs have the ability to engraft and possess the most stemness, while the CD90- CD34+ MPPs cannot engraft but retain the ability to form colonies and the CD34- cells are unable to form colonies (Doulatov et al., 2012). A loss of CD34 is therefore considered a sign of differentiation and loss of primitiveness (Baudet et al., 2016). Using the same CD34+ model we could also study the effect of H4 alone and an increased differentiation was observed following treatment, showing that H4 affects healthy HSPCs as well. This means that we now have a stem cell model that responds to both IL3 and H4. This would also be possible in the primary AML sample, but since we have a limited supply of that sample it is of interest to have an alternative model system and use the primary AML sample to validate the results.

To assess if H4 potentiated the effect of IL3, the effect of a combination of 5µM H4 and changing IL3 concentration was studied. The resulting decrease in MPPs was normalized to non-IL3 treated cells with and without H4 for the corresponding populations to account for the effect of H4 alone. We observed that H4 had a potentiating effect on the IL3 sensitivity, as the IL3-dependent decrease of CD34+ cells was greater when the cells were treated with H4. This supports the hypothesis that H4 increases IL3 sensitivity, but additional biological replicates are required to show whether the change is statistically significant. Cord blood derived cells lack the malignant state of the AML cell line and primary sample, which could have compromised intracellular signaling, and this will impact the accuracy of the cord blood model for our study of H4. Any results obtained using this model will therefore need to be validated on primary AML cells, however this
can also be stated for results obtained using the cell line due to the previously discussed differences between the two cell types.

As the experiments with primary cells lacked biological replicates due to time constraints and limited resources, many of them (such as the cell cycle assay) would have to be repeated in order to draw reliable conclusions. Further testing will also be required to understand if treatment with H4 does indeed lead to an increased IL3 sensitivity and signaling. To accomplish this, we could look at the effect of H4 on the activation/increase of the signaling pathways downstream of the IL3-receptor such as the JAK/STAT pathway and the ERK-pathway where c-Raf is located in a setting with and without IL3. Such an experiment was planned using Western blot to assess protein phosphorylation levels of STAT5 and p38 respectively, but this was abandoned due to time constraints. Another strategy to examine the potential connection between H4 treatment and increased expression of CD123 could be to block translation in the cells prior to H4 treatment and look at whether the expression of mRNA for the IL3 receptor is increased. This could show whether the upregulation is a direct effect of H4 or a consequence of another unknown factor affected by H4. It would also be of interest to study response to H4-treatment in AML cells with and without constitutive expression of the IL3-receptor (CD123) to observe if treatment further increases the expression and thereby the sensitivity to IL3, and if this increases the differentiation response.

IL3 alone has been shown to induce cycling in AML blast cells, and blocking of the IL3-receptor by targeting CD123 inhibited engraftment of leukemic stem cells (LSCs) in in vivo models (Jin et al., 2009). This strategy of blocking CD123 is an example of targeting LSCs, but not induce differentiation in them, and one can argue if this is a long term solution since the LSCs could still survive in their quiescent state in the same way that they do during chemotherapy. However, the knowledge that IL3 induces cycling in AML blast cells could indicate that upregulation of the IL3-receptor is an early step in the mechanism of action of H4 as we know that H4 also has an effect on differentiation independent of IL3. We can also speculate that if H4 does mediate its effect by increasing IL3 sensitivity, this could indicate that treatment with H4 restores normal IL3-signaling in
the same way that the ATRA differentiation therapy restores normal retinoic acid signaling (Marchwicka et al., 2014).

In future clinical applications it is of therapeutical importance to have a method for assessing whether or not a patient will respond to a certain therapy. By studying a connection between the expression of CD123 and sensitivity to H4-treatment in the AML cells, this knowledge could be used to design a scoring system for how likely a patient is to respond to this particular therapy. In a long term perspective this could help improve survival rates among AML patients, however plenty of further testing is required prior to clinical application of our results.

**Experimental Procedures**

*Cell culture and compounds*

The U937 (DSMZ, Cat#: ACC-5) and Mono-Mac 6 (MM6) (a gift of Dr Marcus Järås) AML cell lines were maintained in RPMI 1640 supplemented with respectively 10% and 15% decomplemented fetal calf serum (FCS). The MM6 culture medium was also supplemented with 1% penicillin/streptomycin.

The primary patient samples (A4 cells) were kept at -150°C prior to culture. The cells were cultured on irradiated OP9M2 stromal cells (Magnusson, Sierra et al. 2013) in α-MEM, supplemented with 20% FCS, 1% penicillin/streptomycin/glutamine, 50 ng/mL of SCF and 25 ng/mL each of TPO, FLT3-L, IL3 and IL6. The cytokines were purchased from Peprotech. The A4 cells were cultured two days before treatment.

Mononuclear cells were purified from cord blood samples using Lymphoprep™ Tubes (Axis-Shield PoC AS, Ref: 1019818). The CD34+ cells were then isolated using CD34 MicroBead Kit (Miltenyi Biotech, Cat#: 130-046-703) and purified twice on MidiMACS columns (Miltenyi Biotech, Cat#: 130-042-301). The isolated CD34+ cells were cultured in SFEM (stem cell technology) supplemented with 100ng/mL each of SCF, TPO, and FLT3-L (Peprotech).

All compounds used in the experiments (H4 and kinase inhibitors) were kept at 10mM in DMSO. Due to this, a corresponding percentage of pure DMSO was used for the non-treated control in each experiment. The kinase inhibitors were purchased from Enzo Life Sciences in solid form.

*FACS analysis*
Equal volumes of the culture were collected to 96 U-shape wells and spin down for 5’ at 350g and 4°C. Pellets were resuspended in staining buffer (PBS without Ca and Mg, supplemented with 2% FCS, 2mM EDTA and specified antibodies) and incubated at 4°C in the dark. Cells were wash once in PBS without Ca and Mg, supplemented with 2% FCS, 2mM EDTA and resuspended in equal volume PBS without Ca and Mg, supplemented with 2% FCS, 2mM EDTA, and SYTOX® Green Nucleic Acid Stain (ThermoFisher, Cat#: S7020) or 7AAD for dead cells exclusion in A4 and U937 respectively. FACS analysis was conducted using FACS Canto II and a volumetric count was used to assess cell count by analyzing the same volume for each sample and counting the number of events following dead cell exclusion.

Antibodies used in immunophenotype staining were CD11b (BioLegend, Cat#: 301310), CD14 (BioLegend, Cat#: 367116), CD15 (BioLegend, Cat#: 323030), CD11b (BioLegend, Cat#: 301308), CD64 (BioLegend, Cat#: 305013), CD117 (BioLegend, Cat#: 313217), CD123 (Becton Dickinson, Cat#: 340445), HLA-DR (BioLegend, Cat#: 307618), CD34 (eBioscience, Ref: 17-0349-42), CD90 (BioLegend, Cat#: 328110), CD33 (BioLegend, Cat#: 303304) and CD11b (BioLegend, Cat#: 301342).

Annexin V staining was done with Annexin V Apoptosis Detection Kit I (BD Biosciences, Cat#: 559763) following manufacturer’s instructions after cell surface staining.

**Cell cycle assay**

Cells were fixated and permeabilized using -20°C 75% EtOH and stored at -20°C. Cells were stained in PBS without Ca and Mg using either Nuclear-ID® Red DNA Stain (Enzo Life Sciences, Cat#: ENZ-52406) or a combination of 7AAD and RNaseA (ThermoFisher, Cat#: AM2269). A third staining protocol was performed using propidium iodide (PI) (Sigma-Aldrich, Cat#: 010M1108) in PBS supplemented with 1% FCS.

Ki67 staining was done with FITC Mouse Anti-Ki-67 Set (BD Biosciences, Cat#: 556026) following manufacturer’s instructions.

**MGG staining**

Cells were immobilized on microscope slides using a cytopsin centrifuge and left to dry. The slides were then stained 5 min in May-Grünwald staining solution (Merck KGaA, Cat#: 1.01424.0500) followed by 15 min in 3% Giemsa staining solution (Merck KGaA, Cat#: 1.09204.0500) and washed twice in MilliQ water for 1 min. After drying, the slides were studied and photographed in a microscope.
Data analysis was conducted using FlowJo 10.2, GraphPad Prism 7.0c, MATLAB R2016b and Microsoft Excel. Statistical significance was assessed using an unpaired t-test in the Prism software.

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References


