

# **New Therapeutic Approaches Targeting MLL-AF9**

## **Leukemias Through Epigenetic Reprogramming**

by

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## **Leukemias Through Epigenetic Reprogramming**

Koç University

Graduate School of Sciences and Engineering

This is to certify that I have examined this copy of a master's thesis by

**Discipulus nomen**

and have found that it is complete and satisfactory in all respects,  
and that any and all revisions required by the final  
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*To my family...*

## **ABSTRACT**

### **New Therapeutic Approaches Targeting MLL-AF9 Leukemias Through Epigenetic Reprogramming**

**Discipulus nomen**

**Master of Science in Biomedical Sciences and Engineering**

**July 24, 2020**

Leukemia is a highly complex disorder of blood and bone marrow and characterized by inhibition of differentiation during hematopoiesis which leads to abnormal cell proliferation. Mixed lineage leukemia (MLL) is a form of acute leukemia that represents poor prognosis and due to chromosomal translocation, resulting in a hyperactive MLL fusion protein. MLL leukemias are largely based on epigenetic irregularities rather than genomic instability. A transcription factor (AF9) that fuses with MLL plays a role in the uncontrolled growth of acute monocytic leukemia. Chromatin modifying enzymes are aberrantly expressed in leukemias and targeting these regulators such as the Lysine-specific demethylase (LSD1) and Histone deacetylase (HDAC) has been considered as a novel treatment modality. In MLL-AF9 leukemias, recruitment of these enzymes due to translocation results in the activation of several genes that inhibit differentiation and lead uncontrolled cell proliferation.

In this study, novel compounds that synthesized to inhibit LSD1, HDAC6, and both LSD1& HDAC6 (Dual Inhibitor) are characterized. We showed that the compounds inhibit target enzymes by in vitro enzyme activity tests, we also showed that the compounds engage with the target enzymes in the cell by cellular thermal shift assay (CETSA). The toxic effects of the compounds on leukemia cells were shown with ATP-dependent cell viability tests. Increased histone methylation and acetylation levels after inhibition of target enzymes were indicated with Western blot. Increased expression of downstream genes due to enzyme inhibition was showed with RT-qPCR. The synergistic effect of the inhibitors with the therapeutic drugs used in the clinic was investigated. We showed that the death response could be increased with a combination of Doxorubicin and our epigenetic inhibitors. Finally, RNA-seq analysis revealed the molecular mechanism of Doxorubicin synergy.

Defects in epigenetic pathways involving increased expression levels or abnormal patterns of activity are one of the key drivers of cell proliferation in cancer. Targeting MLL-AF9 is an attractive strategy for therapeutic intervention in patients with this genetic translocation. Since our inhibitors are more effective on MLL-AF9 leukemias, we hope that our study will result in the characterization of targeted drugs for use in leukemia therapy.



## ÖZETÇE

### Epigenetik Yeniden Programlama ile MLL-AF9 Lösemilerini

### Hedefleyen Yeni Tedavi Yöntemleri

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Lösemi, oldukça karmaşık bir kan ve kemik iliği bozukluğudur ve hematopoez sırasında anormal hücre proliferasyonuna yol açan farklılaşmanın inhibisyonu ile karakterize edilmektedir. Karışık soy lösemileri (MLL), kötü prognozu temsil eden ve kromozomal translokasyona bağlı olarak hiperaktif bir MLL füzyon proteini ile sonuçlanan bir akut lösemi türüdür. MLL lösemileri büyük ölçüde genomik instabilite yerine epigenetik düzensizliklere dayanır. MLL ile kaynaşan bir transkripsiyon faktörü (AF9), akut monositik lösemisinin kontrolsüz büyümesinde rol oynamaktadır. Kromatin değiştirici enzimler, lösemilerde anormal şekilde eksprese edilmektedir. Lizin spesifik demetilaz (LSD1) ve Histon deasetilaz (HDAC) gibi bu düzenleyicilerin hedeflenmesi yeni bir tedavi yöntemi olarak kabul edilmiştir. MLL-AF9 füzyonunda kromozomal translokasyon nedeniyle bu enzimlerin interaksyonu, farklılaşmayı engelleyen ve kontrolsüz hücre çoğalmasına neden olan birkaç genin aktivasyonuna yol açmaktadır.

Bu çalışmada, LSD1, HDAC6 ve hem LSD1 & HDAC6'yı (Çift İnhibitör) inhibe etmek için sentezlenen yeni bileşikler AML hücrelerinde karakterize edilmektedir. Çalışmamızda; bileşiklerin in vitro enzim aktivite testleri ile hedef enzimleri inhibe ettikleri gösterilmiş, ayrıca bileşiklerin hücresel termal kayma deneyi (CETSA) ile hedef enzimleri hücre içinde de bağlanabildikleri gösterilmiştir. Bileşiklerin lösemi hücreleri üzerindeki toksik etkileri ATP'ye bağlı hücre canlılık testleriyle gösterilmiştir. Hedef enzimlerin inhibisyonundan sonra artan histon metilasyonu ve asetilasyon seviyeleri Western blot ile gösterilmiştir. Enzim inhibisyonuna bağlı olarak hedef genlerinin artan ekspresyonu RT-qPCR ile gösterilmiştir. İnhibitörlerin klinikte kullanılan terapötik ilaçlarla sinerjistik etkisi araştırılmıştır ve AML büyümesinin Doksorubisin ve epigenetik

inhibitörlerimizle durdurulabildiği gösterilmiştir. Son olarak, RNA-dizileme analizi Doksorubisin sinerjisinin moleküler mekanizmasını ortaya çıkarmıştır.

Artmış ekspresyon seviyelerini veya anormal aktivite modellerini içeren epigenetik yollardaki kusurlar, kanserdeki hücre proliferasyonunun temel faktörlerinden biridir. MLL-AF9'un hedeflenmesi, bu genetik translokasyonu olan hastalarda terapötik müdahale için çekici bir stratejidir. İnhibitörlerimiz MLL-AF9 lösemiler üzerinde daha etkili olduğundan, çalışmamızın lösemi tedavisinde kullanılmak üzere hedeflenen ilaçların karakterizasyonu ile sonuçlanmasını umuyoruz.



## TABLE OF CONTENTS

|                                                                     |      |
|---------------------------------------------------------------------|------|
| List of Tables .....                                                | xiii |
| List of Figures.....                                                | xiv  |
| Abbreviations.....                                                  | xvii |
| Chapter 1: INTRODUCTION .....                                       | 1    |
| 1.1    Epigenetics.....                                             | 1    |
| 1.1.1    DNA Methylation/Demethylation .....                        | 1    |
| 1.1.2    Chromatin Remodeling .....                                 | 2    |
| 1.1.3    Histone Methylation/Demethylation .....                    | 3    |
| 1.1.4    Histone Acetylation/Deacetylation.....                     | 4    |
| 1.1.5    Histone Phosphorylation/Dephosphorylation.....             | 6    |
| 1.1.6    Histone Ubiquitylation/Deubiquitylation .....              | 7    |
| 1.1.7    Recognition of Histone Modifiers .....                     | 8    |
| 1.2    Leukemia .....                                               | 9    |
| 1.2.1    Acute Myeloid Leukemia .....                               | 9    |
| 1.2.2    Chromosomal Translocations in Acute Myeloid Leukemia.....  | 10   |
| 1.3    MLL-Rearranged Leukemias.....                                | 12   |
| 1.3.1    MLL1.....                                                  | 12   |
| 1.3.2    MLL-AF9 Fusions.....                                       | 13   |
| 1.4    Molecular and Epigenetic Mechanisms of MLL-AF9 Fusions ..... | 15   |
| 1.4.1    LSD1.....                                                  | 16   |
| 1.4.2    LSD1 Inhibition in MLL-AF9 Leukemias .....                 | 19   |
| 1.4.3    HDACs .....                                                | 22   |
| 1.4.4    HDAC6 Inhibition in Hematologic Malignancies.....          | 24   |
| 1.5    Chemotherapy Regimens in AML .....                           | 26   |
| 1.5.1    Doxorubicin.....                                           | 26   |

|                                       |                                                                            |    |
|---------------------------------------|----------------------------------------------------------------------------|----|
| 1.5.2                                 | Cytarabine.....                                                            | 27 |
| 1.5.3                                 | Other Anti-Cancer Agents .....                                             | 28 |
| 1.6                                   | The Importance of Novel Epigenetic Therapies in AML .....                  | 30 |
| Chapter 2: MATERIALS AND METHODS..... |                                                                            | 32 |
| 2.1                                   | Design and Synthesis of Novel LSD1/HDAC6/Dual Inhibitors .....             | 32 |
| 2.2                                   | Cell Lines and Reagents .....                                              | 33 |
| 2.3                                   | Measuring LSD1 Activity by In-vitro Enzyme Assays .....                    | 33 |
| 2.4                                   | Measuring the Activity of Different HDACs via In-vitro Enzyme Assays<br>34 |    |
| 2.5                                   | Cellular Thermal Shift Assay .....                                         | 34 |
| 2.6                                   | Measurement of LSD1 Expression in Different Cells Lines .....              | 35 |
| 2.6.1                                 | Cell Culture .....                                                         | 35 |
| 2.6.2                                 | Protein Extraction .....                                                   | 35 |
| 2.6.3                                 | Measuring Protein Concentration.....                                       | 36 |
| 2.6.4                                 | SDS-PAGE and Western Blotting.....                                         | 36 |
| 2.7                                   | Cell Viability Tests upon Treatment with Tranylcypromine.....              | 36 |
| 2.7.1                                 | Drug Treatment .....                                                       | 36 |
| 2.7.2                                 | Cell Viability Tests with SRB .....                                        | 36 |
| 2.8                                   | Cell Viability Tests upon Treatment with Novel Inhibitors .....            | 37 |
| 2.9                                   | H3K4 and H3K9 methylation Levels upon LSD1 Inhibition .....                | 37 |
| 2.9.1                                 | Drug Treatment .....                                                       | 37 |
| 2.9.2                                 | Histone Extraction .....                                                   | 37 |
| 2.9.3                                 | Measuring Protein Concentration.....                                       | 38 |
| 2.9.4                                 | SDS-PAGE Gel Electrophoresis and Western Blotting .....                    | 38 |
| 2.10                                  | Acetylated $\alpha$ -tubulin Levels upon HDAC6 Inhibition.....             | 38 |
| 2.10.1                                | Drug Treatment .....                                                       | 38 |
| 2.10.2                                | Protein Extraction .....                                                   | 38 |

|        |                                                                     |    |
|--------|---------------------------------------------------------------------|----|
| 2.10.3 | Measuring Protein Concentration.....                                | 38 |
| 2.10.4 | SDS-PAGE Gel Electrophoresis and Western Blotting .....             | 38 |
| 2.11   | Macrophage Marker Expressions upon LSD1 Inhibition .....            | 39 |
| 2.11.1 | Drug Treatment .....                                                | 39 |
| 2.11.2 | RNA Extraction .....                                                | 39 |
| 2.11.3 | cDNA Synthesis .....                                                | 39 |
| 2.11.4 | RT-qPCR .....                                                       | 40 |
| 2.12   | P21 Gene Expression Levels upon HDAC6 Inhibition .....              | 40 |
| 2.12.1 | Drug Treatment .....                                                | 40 |
| 2.12.2 | RNA Extraction .....                                                | 40 |
| 2.12.3 | cDNA Synthesis .....                                                | 41 |
| 2.12.4 | RT-qPCR .....                                                       | 41 |
| 2.13   | Cell Viability Test with Combination Assays .....                   | 41 |
| 2.13.1 | Drug Treatment .....                                                | 41 |
| 2.13.2 | Cell Viability Tests.....                                           | 41 |
| 2.14   | Determination of Cell Death Mechanism upon Combination Therapy .... | 41 |
| 2.14.1 | Drug Treatment .....                                                | 41 |
| 2.14.2 | Annexin-V Staining.....                                             | 42 |
| 2.14.3 | Caspase 3/7 Staining.....                                           | 42 |
| 2.14.4 | $\gamma$ H2AX Activation.....                                       | 42 |
| 2.14.5 | Reactive Oxygen Species Activation .....                            | 42 |
| 2.15   | DNA Repair Genes Expression upon Inhibitor Treatment.....           | 43 |
| 2.15.1 | Drug Treatment .....                                                | 43 |
| 2.15.2 | RNA Extraction .....                                                | 43 |
| 2.15.3 | cDNA Synthesis .....                                                | 43 |
| 2.15.4 | RT-qPCR .....                                                       | 43 |
| 2.16   | RNA-seq .....                                                       | 44 |

|                          |                                                                                                      |    |
|--------------------------|------------------------------------------------------------------------------------------------------|----|
| 2.16.1                   | Drug Treatment .....                                                                                 | 44 |
| 2.16.2                   | RNA Extraction .....                                                                                 | 44 |
| 2.16.3                   | RNA-Sequencing and Analysis .....                                                                    | 44 |
| 2.17                     | Validation of RNA-seq Hit Genes by RT-qPCR .....                                                     | 45 |
| 2.17.1                   | cDNA Synthesis .....                                                                                 | 45 |
| 2.17.2                   | RT-qPCR .....                                                                                        | 45 |
| 2.18                     | Statistical Analysis.....                                                                            | 47 |
| Chapter 3: RESULTS ..... |                                                                                                      | 48 |
| 3.1                      | Synthesis of Novel Inhibitors .....                                                                  | 48 |
| 3.1.1                    | AD-94 is a Novel LSD1 Inhibitor .....                                                                | 48 |
| 3.1.2                    | MH-25 is a Novel HDAC6 Inhibitor .....                                                               | 49 |
| 3.1.3                    | AD-58 is a Novel LSD1/HDAC6 Dual Inhibitor .....                                                     | 50 |
| 3.2                      | In-vitro Enzyme Activities of Novel Inhibitors .....                                                 | 50 |
| 3.3                      | Inhibitors Stabilize Target Enzymes in THP-1 cells: CETSA.....                                       | 52 |
| 3.4                      | LSD1 is Overexpressed in Leukemia .....                                                              | 54 |
| 3.5                      | Tranylcypromine Significantly Reduces Growth of AML .....                                            | 55 |
| 3.6                      | Pharmacological LSD1 and HDAC6 Inhibition Induced Cell Death in<br>Leukemia Cells .....              | 56 |
| 3.7                      | Pharmacological Inhibition of LSD1 Induces Global H3K Methylation .                                  | 59 |
| 3.8                      | Pharmacological Inhibition of HDAC6 Induces Acetylated $\alpha$ -Tubulin ...                         | 61 |
| 3.9                      | LSD1 Inhibition Induces Macrophage Markers Expression.....                                           | 63 |
| 3.10                     | The Cyclin-dependent Kinase Inhibitor p21 Expressions Upon<br>Pharmacological HDAC6 Inhibition ..... | 64 |
| 3.11                     | Combination of Novel Inhibitors with Clinically Used Agents .....                                    | 65 |
| 3.12                     | Pharmacological LSD1 and HDAC6 inhibition with Doxorubicin Induces<br>Apoptosis in AML cells.....    | 68 |
| 3.12.1                   | Annexin V Staining .....                                                                             | 68 |
| 3.12.2                   | Caspase-3/7 Staining .....                                                                           | 71 |

|                              |                                                                                    |     |
|------------------------------|------------------------------------------------------------------------------------|-----|
| 3.13                         | Do the Inhibitors Synergize with Clinically Used Analogues? .....                  | 73  |
| 3.14                         | How Does the Synergy Work? .....                                                   | 75  |
| 3.14.1                       | Assessment of Synergy through Topoisomerase II Inhibition Function<br>75           |     |
| 3.14.2                       | ROS Generation Profile upon Combination Treatment .....                            | 78  |
| 3.14.3                       | Assessment of Screening DNA Damage Response Following<br>Inhibitor Treatment ..... | 80  |
| 3.15                         | RNA-seq Analysis .....                                                             | 82  |
| 3.16                         | Gene Ontology and Pathway Enrichment Analysis.....                                 | 93  |
| 3.17                         | Validation of RNA-seq Hit Genes by RT-qPCR.....                                    | 95  |
| Chapter 4: DISCUSSION .....  |                                                                                    | 98  |
| 4.1                          | LSD1 Inhibition .....                                                              | 98  |
| 4.2                          | HDAC6 Inhibition .....                                                             | 105 |
| 4.3                          | iDual Inhibition.....                                                              | 107 |
| Chapter 5: CONCLUSIONS ..... |                                                                                    | 111 |
| Bibliography .....           |                                                                                    | 112 |

## LIST OF TABLES

|                                                                                                                                                                |    |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| <b>Table 1.1:</b> Diagnostic fusion gene detection frequencies in ALL and AML's prognosis.....                                                                 | 14 |
| <b>Table 2.1:</b> Macrophage marker genes and primer sequences. ....                                                                                           | 40 |
| <b>Table 2.2:</b> p21 specific RT-qPCR primers. ....                                                                                                           | 41 |
| <b>Table 2.3:</b> DNA repair genes and specific primer sequences. ....                                                                                         | 43 |
| <b>Table 2.4:</b> RNA-seq hit Differentially expressed genes and specific primer sequences. ....                                                               | 45 |
| <b>Table 3.1:</b> IC <sub>50</sub> values of novel inhibitors and positive control GSK2879552 for LSD1, HDAC6, HDAC1, and HDAC3 in kinetic enzyme assays. .... | 51 |
| <b>Table 3.2:</b> Half inhibitory concentration (IC <sub>50</sub> ) values of inhibitors on four different leukemia lines .....                                | 58 |
| <b>Table 3.3:</b> Table showing differentially expressed LSD1-related genes, upon AD-94 treatment. ....                                                        | 86 |
| <b>Table 3.4:</b> Table showing differentially expressed HDAC6-related genes, upon MH-25 treatment.....                                                        | 89 |
| <b>Table 3.5:</b> Table showing differentially expressed monocyte-macrophage differentiation related-genes, upon both AD-94 and AD-58 treatment. ....          | 90 |
| <b>Table 3.6:</b> Table showing differentially expressed Doxorubicin sensitizer-genes, upon AD-94, MH-25, and AD-58 treatment.....                             | 91 |
| <b>Table 3.7:</b> Table showing other differentially expressed gene families, upon AD-94, MH-25, and AD-58 treatment.....                                      | 92 |

## LIST OF FIGURES

|                                                                                                                                                     |    |
|-----------------------------------------------------------------------------------------------------------------------------------------------------|----|
| <b>Figure 1.1:</b> Histone Methyl Transferase and Demethylase enzymes in humans.....                                                                | 4  |
| <b>Figure 1.2:</b> Histone Acetyl Transferase and Deacetylase enzymes in human.....                                                                 | 5  |
| <b>Figure 1.3:</b> Classification of HAT and HDAC enzymes in human .....                                                                            | 6  |
| <b>Figure 1.4:</b> Known histone phosphorylation .....                                                                                              | 7  |
| <b>Figure 1.5:</b> Known histone ubiquitylation.....                                                                                                | 8  |
| <b>Figure 1.6:</b> Major classification in leukemia .....                                                                                           | 9  |
| <b>Figure 1.7:</b> Structure of LSD1 enzyme and histone demethylation reaction .....                                                                | 18 |
| <b>Figure 1.8:</b> The role of LSD1 enzyme in transcriptional regulation .....                                                                      | 19 |
| <b>Figure 1.9:</b> Chemical structures of LSD1 inhibitors .....                                                                                     | 22 |
| <b>Figure 1.10:</b> Structure of human HDAC6 enzyme .....                                                                                           | 23 |
| <b>Figure 1.11:</b> Chemical structures of HDAC6 inhibitors.....                                                                                    | 26 |
| <b>Figure 2.1:</b> Synthesis reaction of AD-58 dual inhibitor .....                                                                                 | 32 |
| <b>Figure 2.2:</b> Synthesis reaction of the AD-94 LSD1 inhibitor.....                                                                              | 33 |
| <b>Figure 3.1:</b> Chemical structure of novel LSD1 inhibitor AD-94 and negative control MH-18 .....                                                | 48 |
| <b>Figure 3.2:</b> Chemical structure of novel HDAC inhibitor MH-25 .....                                                                           | 49 |
| <b>Figure 3.3:</b> Chemical structure of novel dual inhibitor AD-58 .....                                                                           | 50 |
| <b>Figure 3.4:</b> Target engagement upon inhibitor treatment in THP-1 cells.....                                                                   | 53 |
| <b>Figure 3.5:</b> LSD1 expression levels in different cancer cell lines and non-cancerous HVT cell line .....                                      | 54 |
| <b>Figure 3.6:</b> LSD1 expression levels in four different leukemia cells .....                                                                    | 55 |
| <b>Figure 3.7:</b> Cytotoxic effects of pharmacological inhibition of LSD1 via non-specific inhibitor Tranylcypromine on different cell lines ..... | 56 |
| <b>Figure 3.8:</b> Cytotoxic effects of novel inhibitors in MLL-fused AML cell lines...                                                             | 57 |
| <b>Figure 3.9:</b> Cytotoxic effects of novel inhibitors in non-MLL-fused CML and T-cell leukemia cell lines.....                                   | 57 |
| <b>Figure 3.10:</b> Increased histone methylation levels upon LSD1 inhibition with Tranylcypromine.....                                             | 59 |
| <b>Figure 3.11:</b> H3K4 dimethylation levels upon LSD1 inhibition with GSK2879552, AD-94, and AD-58 in THP-1 cells .....                           | 60 |

|                                                                                                                                                                     |    |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| <b>Figure 3.12:</b> H3K4 dimethylation levels upon LSD1 inhibition with GSK2879552, AD-94, and AD-58 in MOLM-13 cells .....                                         | 61 |
| <b>Figure 3.13:</b> Acetylated $\alpha$ -tubulin levels upon pharmacological inhibition of HDAC6 .....                                                              | 62 |
| <b>Figure 3.14:</b> Macrophage marker expression upon LSD1 inhibition .....                                                                                         | 63 |
| <b>Figure 3.15:</b> p21 expression levels in THP-1 cells after HDAC6 inhibition.....                                                                                | 64 |
| <b>Figure 3.16:</b> Cell viability in THP-1 cells after combination therapy with novel inhibitors and clinically used agents .....                                  | 68 |
| <b>Figure 3.17:</b> Analysis of Annexin V positive cells by flow cytometry after combination therapy .....                                                          | 69 |
| <b>Figure 3.18:</b> Flow cytometric analysis of the activated caspase-3/7 and simultaneous detection of dead cells by 7-AAD labeling.....                           | 72 |
| <b>Figure 3.19:</b> Cell viability in THP-1 cells after combination Doxorubicin with GSK2879552 and Ricolinostat .....                                              | 74 |
| <b>Figure 3.20:</b> Cell viability in THP-1 cells combined with Etoposide and novel inhibitors .....                                                                | 75 |
| <b>Figure 3.21:</b> Flow cytometric analysis of the $\gamma$ H2AX positive cells.....                                                                               | 77 |
| <b>Figure 3.22:</b> Ros generation profile upon treatment in THP-1 cells .....                                                                                      | 79 |
| <b>Figure 3.23:</b> DNA damage repair gene expressions upon inhibitor treatment .....                                                                               | 81 |
| <b>Figure 3.24:</b> MDS plot reflecting transcriptomic similarity after different treatment groups.....                                                             | 83 |
| <b>Figure 3.25:</b> Venn diagrams showing the number of differentially expressed genes after inhibitor treatments.....                                              | 84 |
| <b>Figure 3.26:</b> Differentially expressed genes upon LSD1 inhibition with AD-94 treatment. ....                                                                  | 85 |
| <b>Figure 3.27:</b> Differentially expressed genes upon HDAC6 inhibition with MH-25 treatment. ....                                                                 | 88 |
| <b>Figure 3.28:</b> Differentially expressed genes upon LSD1/HDAC6 inhibition with AD-58 treatment. ....                                                            | 90 |
| <b>Figure 3.29:</b> Gene ontology and pathway enrichment analysis upon LSD1/HDAC6 inhibition.....                                                                   | 95 |
| <b>Figure 3.30:</b> RT-qPCR analysis of top differentially expressed genes for validation of RNA-Seq hits upon LSD1 inhibition with AD-94 and AD-58 treatment. .... | 96 |

**Figure 3.31:** RT-qPCR analysis of top differentially expressed genes for validation of RNA-Seq hits upon HDAC6 inhibition with MH-25 and AD-58 treatment..... 96



## ABBREVIATIONS

|          |                                                     |
|----------|-----------------------------------------------------|
| 7-AAD    | 7-Aminoactinomycin D                                |
| AF       | ALL1-Fused gene from chromosome                     |
| AD-58    | LSD1/HDAC6 Inhibitor Novel Compound                 |
| AD-94    | LSD1 Inhibitor Novel Compound                       |
| AGPAT9   | Glycerol-3-phosphate acyltransferase 3              |
| AIM1     | Absent in melanoma 1                                |
| ALL      | Acute Lymphoid Leukemia                             |
| ALOX5    | Arachidonate 5-Lipoxygenase                         |
| AML      | Acute Myeloid Leukemia                              |
| ANXA2    | Annexin 2A                                          |
| AOL      | Amine Oxidase Like                                  |
| APL      | Acute Promyelocytic Leukemia                        |
| AR       | Androgen Receptor                                   |
| ARHGAP26 | Rho GTPase Activating Protein 26                    |
| Ash2L    | Set1/Ash2 Histone methyltransferase complex subunit |
| ATCC     | American Type Culture Collection                    |
| ATF2     | Activating Transcription Factor 2                   |
| ATM      | Ataxia-telangiectasia Mutated                       |
| ATR      | ATM and Rad3-related                                |
| ATRA     | All-Trans Retinoic Acid                             |
| BCA      | Bicinchoninic Acid                                  |
| BCR      | Breakpoint Cluster Region                           |
| BD       | Bromodomain                                         |
| BET      | Bromodomain Extra-terminal                          |
| BER      | Base Excision Repair                                |
| BRCT     | BRCA1 C Terminus                                    |
| BRD      | Bromodomain                                         |
| BRD4     | Bromodomain-containing 4                            |
| BSA      | Bovine Serum Albumin                                |
| BTG1     | B-cell Translocation Gene 1                         |
| C-       | Carboxyl                                            |
| C-Ara    | Cytosine Arabinoside                                |

|                    |                                                        |
|--------------------|--------------------------------------------------------|
| CAMSAP2            | Calmodulin Regulated Spectrin Protein Family Member 2  |
| CBF                | Core-Binding Factor                                    |
| CBP                | CREB-binding protein                                   |
| CBP/p300           | Histone acetyltransferase                              |
| CCDC109B           | Coiled-coil Domain Containing 109B                     |
| CD                 | Cluster of Differentiation / Catalytic Domain          |
| cDNA               | Complementary Deoxyribonucleic Acid                    |
| CDK                | Cyclin-dependent Kinases                               |
| Ch-IP              | Chromatin immunoprecipitation                          |
| CIS                | Cisplatin                                              |
| CLEC5A             | C-Type Lectin Domain Containing 5A                     |
| CLL                | Chronic Lymphoid Leukemia                              |
| CML                | Chronic Myeloid Leukemia                               |
| COX-2              | Cyclooxygenase-2                                       |
| CTG                | Cell Titer Glo                                         |
| CYT                | Cytarabine                                             |
| ddH <sub>2</sub> O | Double Distilled Water                                 |
| DDIT4              | DNA Damage Inducible Transcript 4                      |
| DEG                | Differentially Expressed Genes                         |
| DNMT1              | DNA methyltransferase 1                                |
| dNTP               | Deoxynucleoside Triphosphates                          |
| DMB                | Dynein Motor Binding                                   |
| DMEM               | Dulbecco's Modified Eagle Medium                       |
| DMSO               | Dimethyl Sulfoxide                                     |
| DNMT               | DNA Methyl Transferase                                 |
| DOT1L              | Disruptor of telomeric silencing 1 protein             |
| DOX                | Doxorubicin                                            |
| DSMZ               | Deutsche Sammlung von Mikroorganismen und Zellkulturen |
| DSB                | Double Strand Break                                    |
| DTX4               | Deltex E3 Ubiquitin Ligase 4                           |
| E2F1               | Retinoblastoma-associated protein 1                    |
| EGFR               | Epidermal Growth Factor Receptor                       |
| ELL                | Elongation Factor for RNA polymerase II                |

|                  |                                          |
|------------------|------------------------------------------|
| ENL              | Eleven-nineteen leukemia                 |
| ER               | Estrogen Receptor                        |
| FAD              | Flavin Adenine Dinucleotide              |
| FBS              | Fetal Bovine Serum                       |
| FCRLA            | Fc Receptor Like A                       |
| FDA              | Food and Drug Administration             |
| FNBP1L           | Formin Binding Protein 1 Like            |
| GCN              | General Control Nondrepressible          |
| GFI              | Growth Factor Independence               |
| GFP              | Green Fluorescent Protein                |
| GLIPR1           | GLI Pathogenesis Related 1               |
| GPR138           | Olfactory Receptor Family 6              |
| $\gamma$ H2AX    | Phosphorylated H2A Histone               |
| H3K4me2          | Histone H3 Lysine 4 Di-methylation       |
| HCl              | Hydrogen Chloride                        |
| HDAC             | Histone Deacetylase                      |
| HDR              | Homology Directed Repair                 |
| HEXB             | Hexosaminidase Subunit Beta              |
| HIF-1 $\alpha$   | Hypoxia Inducible Factor 1               |
| HIPK2            | Homeodomain Interacting Protein Kinase 2 |
| HMT              | Histone Methyl Transferase               |
| hMOF             | Histone Acetyltransferase                |
| HRP              | Horse Radish Peroxidase                  |
| HSP              | Heat Shock Protein                       |
| HVT              | Human Villous Trophoblasts               |
| IC <sub>50</sub> | Inhibitory Half Concentration            |
| IFI44            | Interferon Induced Protein 44            |
| IFI44L           | Interferon Induced Protein 44 Like       |
| IL10RA           | Interleukin 10 Receptor Subunit Alpha    |
| ITGAM            | Integrin Subunit Alpha M                 |
| IQGAP2           | Ras GTPase-activating-like protein       |
| JMJD             | Jumanji Domain                           |
| JNK              | c-Jun N-terminal kinases                 |

|        |                                                        |
|--------|--------------------------------------------------------|
| KAT    | Lysine Acetyl Transferase                              |
| KCTD12 | Potassium Channel Tetramerization Domain Containing 12 |
| KDM1A  | Lysine [K]-specific Demethylase 1                      |
| KMT2A  | Lysine [K]-specific Methyl Transferase 2A              |
| logFC  | Logarithmic Fold Change                                |
| LPXN   | Leupaxin                                               |
| LRP1   | LDL Receptor Related Protein 1                         |
| LSD1   | Lysine-Specific Demethylase 1                          |
| LY96   | Lymphocyte Antigen 96                                  |
| MBD    | Menin-binding domain                                   |
| MBT    | Malignant Brain Tumor                                  |
| MDC-1  | Mediator of DNA Damage Checkpoint 1                    |
| MDR1   | Multi Drug Resistance                                  |
| MDS    | Multidimensional plot                                  |
| MH-18  | Negative Control Novel Compound                        |
| MH-25  | HDAC6 Inhibitor Novel Compound                         |
| MLL    | Mixed-Lineage Leukemia                                 |
| MLL1   | Mixed-Lineage Leukemia gene                            |
| MPEG1  | Macrophage Expressed 1                                 |
| N-     | Amino                                                  |
| NAD    | Nicotinamide Adenine Dinucleotide                      |
| NaOH   | Sodium Hydroxide                                       |
| NHEJ   | Non-Homologous Ends                                    |
| NER    | Nucleotide Excision Repair                             |
| NES    | Nuclear Exclusion Site                                 |
| NUP98  | Nuclear Pore Complex Protein                           |
| OAS    | 2'-5'-Oligoadenylate Synthetase                        |
| OSBP1L | Oxysterol-binding protein 1 Like                       |
| P53    | TP53 Tumour Suppressor                                 |
| PAGE   | Poly Acrylamide Gel Electrophoresis                    |
| PADI   | Peptidyl arginine deiminase                            |
| PBS    | Phosphate Buffer Saline                                |
| PCTXL  | Paclitaxel                                             |

|         |                                                  |
|---------|--------------------------------------------------|
| PDGFR   | Platelet Derived Growth Factor                   |
| PDX     | Patient Derived Xenografts                       |
| PHD     | Plant Homeodomain fingers                        |
| Pi      | Protease Inhibitory Cocktail                     |
| PLBD1   | Phospholipase B Domain Containing 1              |
| PLZF    | Promyelocytic Leukemia Zinc Finger               |
| PML     | Promyelocytic Leukemia                           |
| PMSF    | Phenyl Methyl Sulfonyl Fluoride                  |
| PS      | Phosphatidylserine                               |
| PTD     | Partial Tandem Duplication                       |
| PTM     | Post Translational Modifications                 |
| PVDF    | Polyvinylidene Difluoride                        |
| RARA    | Retinoic Acid Receptor Alpha                     |
| RbBP5   | Retinoblastoma-binding Protein 5                 |
| RD      | Repression Domains                               |
| RGMB    | Repulsive Guidance Molecule BMP Co-Receptor B    |
| rDNase  | Recombinant DNase Enzyme                         |
| RIPA    | Radioimmunoprecipitation Assay                   |
| RNA     | Ribonucleic Acid                                 |
| ROS     | Reactive Oxygen Species                          |
| RT-qPCR | Real Time Quantitative Polymerase Chain Reaction |
| S100A12 | S100 calcium-binding protein A12                 |
| SAM     | S-adenosyl Methionine                            |
| SAP     | Stress Activating Protein                        |
| SASH1   | SAM And SH3 Domain Containing 1                  |
| SCPEP1  | Serine Carboxypeptidase 1                        |
| SDS     | Sodium Dodecyl Sulfate                           |
| SE14    | Serine/glutamate-rich repeat                     |
| SET     | H3K4 histone methyltransferase domain            |
| SEC     | Super Elongation Complex                         |
| shRNA   | Short-hairpin RNA                                |
| SLC7A7  | Solute Carrier Family 7 Member 7                 |
| SIRT    | Sirtuin                                          |

|       |                                                    |
|-------|----------------------------------------------------|
| SNL   | Speckled Nuclear Localization                      |
| SNX7  | Sorting Nexin 7                                    |
| STAT3 | Signal Transducer and Activator of Transcription 3 |
| SRB   | Sulforhodamine B                                   |
| SYK   | Spleen Tyrosine Kinase                             |
| TAD   | Transactivation Domain                             |
| TCP   | Tranylcypromine                                    |
| TDRD3 | Tudor Domain Containing 3                          |
| TEB   | Triton Extraction Buffer                           |
| TET   | Ten-Eleven Translocation                           |
| Topo  | Topoisomerase                                      |
| UBD   | Ubiquitin Binding Domain                           |
| UV    | Ultraviolet                                        |
| WBC   | White Blood Cells                                  |
| WDR5  | WD Repeat-containing Protein 5                     |
| ZNF   | Zinc Finger                                        |

## Chapter 1: INTRODUCTION

### **1.1    *Epigenetics***

Epigenetic changes alter the physical structure of DNA and ensure that genetic information is inherited without causing alterations in the DNA sequence [Lund and van Lohuizen, 2004]. Modifications, which include the addition or removal of chemical groups on DNA or histones or histones or other macromolecules may affect gene activity and determine which genes should be turned on or off. Epigenetic modifications remain as cells divide; therefore, are inherited. They may take place either directly or indirectly, regulating chromatin structure, leading to gene expression changes. Epigenetic regulatory enzymes, including DNA methyltransferases (DNMT's), histone methyltransferases (HMT's), and histone deacetylases (HDAC's) are major proteins that catalyze these enzymatic reactions [Han et al., 2019]. The enzymes can be classified as writers or erasers based on whether they add or remove the marks on these macromolecules, respectively. The third group of enzymes is called readers, which provide the interpretation of these epigenetic marks added or removed by the writers or the erasers. In this section, different types of epigenetic modifications and the players involved in these changes will be discussed in detail.

#### **1.1.1    *DNA Methylation/Demethylation***

One of the major epigenetic changes that regulate gene expression is DNA methylation, which is a highly effective mechanism to silence specific regions on the genome. The covalently modified changes on the DNA is copied during replication, thus the expression state is inherited to the offspring until a new stimulus for removal of that mark [Ballestar and Esteller, 2002]. DNA methylation usually occurs at cytosine residues in 5'-CpG-3' dinucleotides, and S-adenosyl methionine (SAM) acts as the methyl donor [Singal and Ginder, 1999]. There are 3 known enzymes responsible for DNA methylation, which can be listed as are DNMT1, DNMT3a, and DNMT3b. While DNMT1 provides methylation from the parent DNA strand to the newly synthesized hemimethylated DNA, DNMT3a and DNMT3b drive *de novo* methylation and do not require a hemimethylated

DNA [Chen et al., 2014]. Usually, methylation of DNA is associated with decreased gene expression since methylated DNA interferes with the binding of transcription factors. It is a major mechanism for controlling various cellular processes such as restriction of cell type-specific expression, control of imprinted genes, or regulation of X-chromosome inactivation [Singal and Ginder, 1999].

Methyl groups on the DNA can be removed through the function of eraser proteins that belong to the ten-eleven translocation (TET) family which hydroxylate the methyl group on cytosine. The decrease in DNA methylation may occur actively or passively, either through direct removal of methyl groups or indirectly by the inhibition of DNMT1 during DNA replication. Active demethylation may take place in different ways, including DNA repair mechanisms such as base excision repair followed by deamination, nucleotide excision repair, and oxidative demethylation. Following sperm penetration of the egg, the paternal genome goes also through a complex demethylation process. Similarly, locus-specific demethylation has been observed on terminally differentiated post-mitotic cells such as neurons [Wu and Zhang, 2010].

### 1.1.2 *Chromatin Remodeling*

Chromatin is the basis for modulating gene expression, and the structure of nucleosomes regulates the binding of transcription factors. Nucleosomes are the basic repeating structural unit of chromatin, which contains eight histone proteins and about 146 base pairs of nucleotides (van Holde, 1988). Chromatin may be present as euchromatin or heterochromatin, based on its availability for access for transcription factors. While heterochromatin is the inactive state, euchromatin represents the major genes and is involved in transcription, therefore it condenses at the beginning of the cell cycle [Tamaru, 2010]. Condensation of chromatin occurs by wrapping DNA into histone proteins. Histones (H1, H2A, H2B, H3, H4) are positively charged, small proteins [Marino-Ramirez et al., 2005]. Histones and DNA bind to each other very tightly, because DNA is negatively charged due to the phosphate groups in its phosphate-sugar backbone. 146 base pairs are wrapped in octamer histone proteins consisting of H2A, H2B, H3, H4. The H1 linker histone maintains integrity between DNA and core histone proteins [Kasinsky et al., 2001]. The H3 and H4 histones have tails protruding from the nucleosome, and modifications on these tails control gene expression by regulating DNA

and histone interaction. Histone modifications are covalent post-translational modifications (PTM), which includes methylation, phosphorylation, acetylation, ubiquitylation etc. The PTMs might impact gene expression by altering chromatin structure or recruiting other histone modifiers. Histone modifications act in diverse biological processes such as transcriptional activation/inactivation, chromosome packaging, DNA damage/repair, and cancer progression [Kuo and Allis, 1998; Leeming et al., 2011].

### *1.1.3 Histone Methylation/Demethylation*

The addition of the methyl groups onto histone tails by Histone methyltransferases (HMT's) is called histone methylation. The number of methyl groups and the position of the amino acids determine the fate of transcription. Methylations usually occur on the amino acids of lysine [K] and arginine [R]. While methylation of H3K4, H3K36, H3K79 are involved in transcriptional activation, methylation of H3K9, H3K27, H4K20 are involved in transcriptional silencing [Hyun et al., 2017]. The most studied lysine methyltransferases in humans are SET, MLL, SYMD, EZH, ASHL, DOT1L, and SUV group of enzymes. Arginine methylation, on the other hand, is usually associated with a more accessible chromatin structure, thus it increases active transcription. Protein arginine methyltransferase (PRMT's) family is responsible for the addition of the methyl groups to the arginine amino acid [Blanc and Richard, 2017].

The removal of methyl groups from histone proteins by histone demethylases (HDMs) is defined as histone demethylation. Known histone lysine demethylases include Lysine-specific demethylase (LSD) family and JMJD family containing the JmjC domain [Shi and Tsukada, 2013]. Peptidyl arginine deiminase (PADI) family is responsible for the removal of the methyl groups from arginine [Morera et al., 2016]. HMTs and HDMs function opposing to keep the transcription order in a specific balance.

## HMT and HDM Enzymes in Human

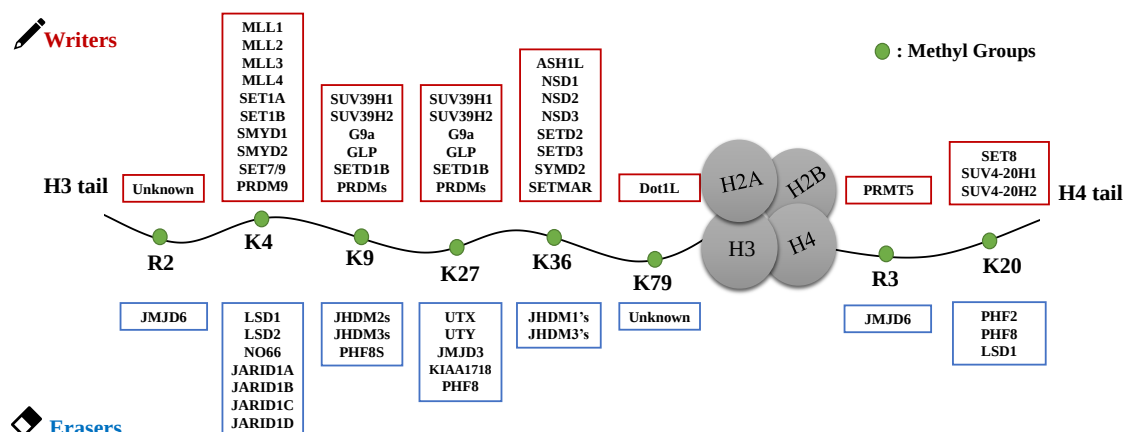


**Figure 1.1:** Histone Methyl Transferase and Demethylase enzymes in humans  
Represented data adapted from Keating and El-Osta, 2015.

### 1.1.4 Histone Acetylation/Deacetylation

Another important covalent modification is the addition or removal of the acetyl groups. Histone acetyltransferases (HATs) use acetyl coenzyme A (acetyl-CoA) for enzymatic addition of an acetyl group ( $\text{COCH}_3$ ) [Maksimoska et al., 2014]. While the addition of the methyl groups may take place on lysine, arginine, and histidine; acetylation takes place most favorably only on lysine. Arginine has 12.5 pK level of the side chain group (highly basic), thus cannot participate in nucleophilic substitution at the carbonyl group of acetyl-CoA. N-Acetylation of imidazole is also not favorable, because it leads to a loss of aromaticity of the imidazole ring in histidine. Lysine acetyltransferases (KATs) control chromatin structure, metabolic activity, and protein stability by the addition of acetyl groups [Montgomery et al., 2015]. Acetylation neutralizes the positive charge on the histones, thereby decreasing the interaction of negatively charged phosphate groups of DNA backbone. Therefore, acetylation increases the binding of transcription factors and gene expression [Verdone et al., 2006]. Activating transcription factor 2 (ATF2), CREB-binding protein (CBP/p300), General Control Nonderepressible (GCNs), and Non-Receptor Tyrosine Kinase (SRCs) are the most conventional acetyltransferase enzymes [Keating and El-Osta, 2015].

Removal of acetyl groups on histones is performed by histone deacetylase enzymes (HDACs). As the acetyl group removed, DNA and histones get closer to each other and

bind more tightly. In contrast to acetylation, deacetylation is associated with decreased transcription [Eberharter and Becker, 2002]. HDACs vary by their subcellular localization (nucleus or cytoplasm), and they are classified into four classes depending on sequence homology [Dokmanovic et al., 2007]. Class I includes HDAC1, HDAC2, HDAC3, HDAC8. Class IIA includes HDAC4, HDAC5, HDAC7, HDAC9. Class IIB includes HDAC6 and HDAC10. Class III contains Sirtuins (SIRT), and class IV includes HDAC11. Sirtuins are  $\text{NAD}^+$  dependent enzymes, while HDACs contain  $\text{Zn}^{+2}$  [Burger and Chory, 2018; Marks and Xu, 2009]. Functional studies revealed that the HDAC1 knock out mice die due to incomplete embryogenesis, and the overexpression of other class I HDACs cannot improve for the loss of HDAC1 [Gallinari et al., 2007]. On the other hand, knock out of HDAC5 and HDAC9 results in a phenotype with cardiac defects [Chang et al., 2004]. Overall, HATs and HDACs are important enzymes involved in gene transcription and their proper function is essential for a healthy transcriptome.

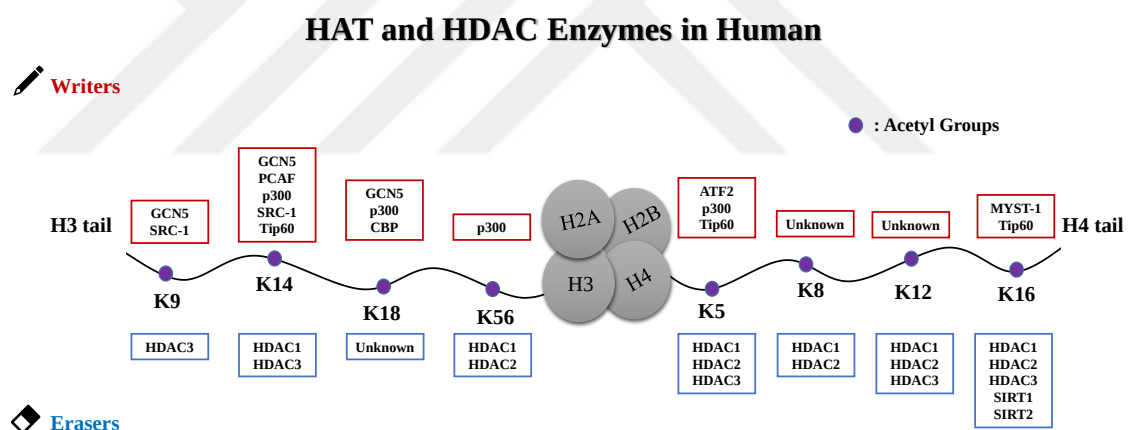


**Figure 1.2:** Histone Acetyl Transferase and Deacetylase enzymes in human

Represented data adapted from Wilting and Dannenberg, 2015.

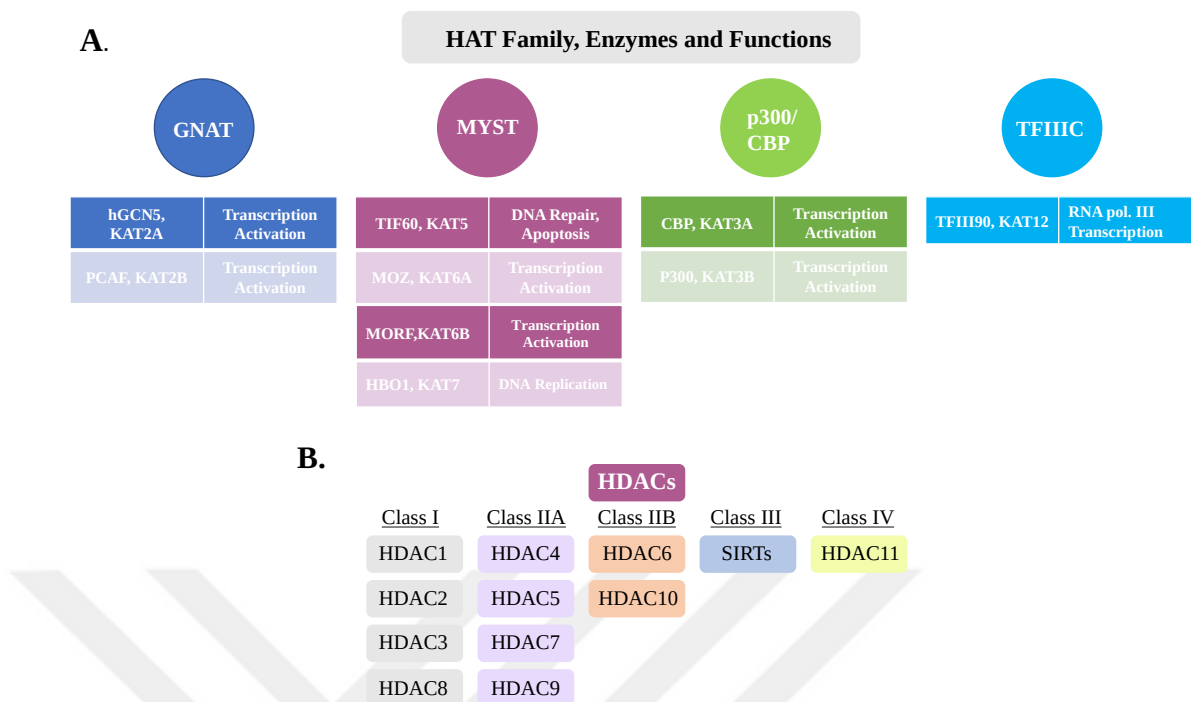


**Figure 1.3:** Classification of HAT and HDAC enzymes in human Major HAT families, enzymes, and functions (**A**), HDAC classification (**B**).

Represented data adapted from Zhou and Zhu, 2009.

### 1.1.5 Histone Phosphorylation/Dephosphorylation

Histone phosphorylation is another known modification and it occurs in many different cellular activities other than chromatin remodeling. Kinases carry out this enzymatic reaction and phosphorylated amino acids are usually serine [S], threonine [T], and tyrosine [Y]. The most studied histone phosphorylation in mammals is the phosphorylation of serine [S], the 139th amino acid found in the tail of H2A histone as a response to DNA damage ( $\gamma$ H2AX) [Rogakou et al., 1998]. Ataxia-telangiectasia mutated (ATM) and ATM and Rad3-related (ATR) protein kinases carry out this phosphorylation [Shroff et al., 2004]. Once the DNA has been repaired  $\gamma$ H2AX must be removed from the chromatin by INO80 chromatin remodeling complexes [van Attikum et al., 2007]. Proliferative genes are known to be regulated by phosphorylated histone residues such as H3S10 and S28 [Rossetto et al., 2012]. H3 phosphorylation is preserved between eukaryotes and thought to be related to chromatin condensation during mitosis and meiosis [Wei et al., 1999]. An apoptotic mammalian cell carries H2BS14 phosphorylation, during condensing its chromatin [Cheung et al., 2003]. While histone

phosphorylation indirectly affects transcription, it plays a direct role in cell division and DNA damage.

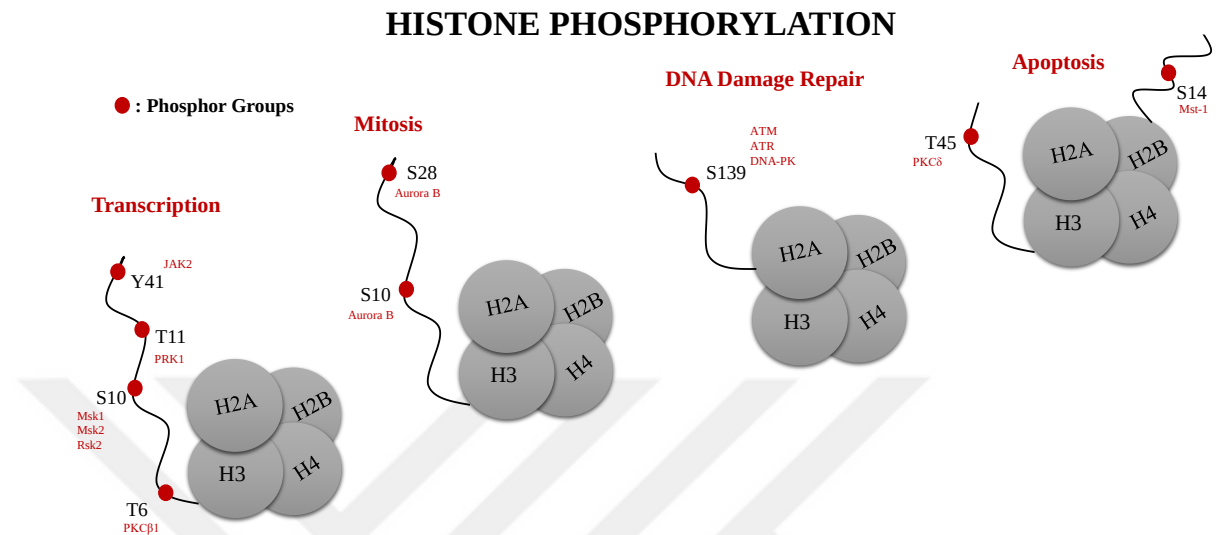


**Figure 1.4:** Known histone phosphorylation

Represented data adapted from Cohen et al., 2011.

#### 1.1.6 Histone Ubiquitylation/Deubiquitylation

Ubiquitin is a regulatory protein found in most tissues in eukaryotes [Kimura and Tanaka, 2010]. Ubiquitylation occurs in three reactions: activation, conjugation, and ligation, performed by ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s), and ubiquitin ligases (E3s), respectively [Pickart and Eddins, 2004]. Ubiquitylation often seems in lysine [K] and methionine [M] groups. H2A and H2B histones are protected from proteasomal degradation by mono-ubiquitylation. Ubiquitylation usually negatively regulates transcription. For instance, H2AK119ub has been studied and found that it participates in Polycomb silencing and X-chromosome inactivation [Wang et al., 2004, Fang et al., 2004]. Ubiquitylation may regulate repression with other modifications via cross-talking. H3K27me3 mark has been known to repress gene expression via H2Aub cross-talking [Kalb et al., 2014]. H2Bub and H3 methylation also crosstalk for regulating DNA damage-induced cell cycle arrest. Similar to H2AX phosphorylation, H2AK13 and K15 ubiquitylation also functions as a signaling marker during DNA damage repair upon double-strand breaks. Both H2Aub and H2Bub have roles in regulating UV lesion repair [Marteijs et al., 2009; Moyal et al., 2011].

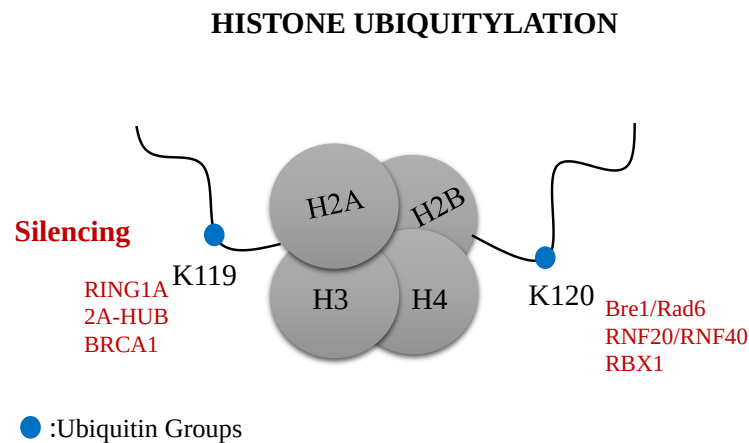


**Figure 1.5:** Known histone ubiquitylation

### 1.1.7 Recognition of Histone Modifiers

A reader protein must recognize histone modifications to direct a transcriptional outcome. There are various protein domains that recognize methylated lysine residues, several of which act through methylation on H3 leading to gene activation or repression [Yap and Zhou, 2011]. For instance, while the activating methylation code on H3 histone is read by the PHD reader domains [Jain et al., 2020], mono and di-methylated lysines on H3 and H4 histone tails are read by The Malignant Brain Tumor (MBT) domains [Bonasio et al., 2010]. Specific H3K4me is recognized by the Tudor domain [Morgan et al., 2017]. Information about methyl arginine readers is limited compared to methyl lysine readers. H3R17me2 and H4R3me2 marks are known to be recognized by the TDRD3 Tudor domain [Yang et al., 2010]. On the other hand, acetyl marks on the lysines are read by bromodomains, which are found in HAT and other chromatin-associated proteins. Bromodomains and bromodomain extra-terminal proteins (BET) include but are not limited to BRD2, BRD3, BRD4. BET proteins direct transcription, in a positive manner. Binding of BET proteins onto a specific locus help initiation and elongation of transcription, because they recruit mediator and elongation complexes [Morgado-Pascual et al., 2019]. Recognition of  $\gamma$ H2AX is mediated by the BRCT domain of Mediator of DNA Damage Checkpoint 1 (MDC-1) [Coster and Goldberg, 2010]. Ubiquitylation must be recognized by the Ubiquitin binding domain (UBD) containing complexes such as BRD4 [Wang et al., 2018]. Therefore, recognition of post-translational modifications by readers is important for the interpretation of chromatin remodeling.

## 1.2 Leukemia

Leukemia is a blood and bone marrow related disease, in which differentiation during the development of blood cells is limited, leading to uncontrolled cell division. Over time, the population of the blood cells with this anomaly increases in the bone marrow and dominates healthy cells, resulting in the signs and symptoms of leukemia. Leukemia is the leading cause of cancer-related deaths among children and adolescents [Harris et al., 2012].

Leukemia, which usually starts in white blood cells, is grouped and named based on how fast it grows (acute or chronic) or on its origin (myeloid or lymphoid) origin [American Cancer Society, 2020]. It may develop through both endogenous causes such as the family history of leukemia and genetic disorders [Horwitz, 1997]; or exogenous causes including previous chemotherapy treatments [Lam, 2007] or exposure the certain chemicals [Vigliani, 1976]

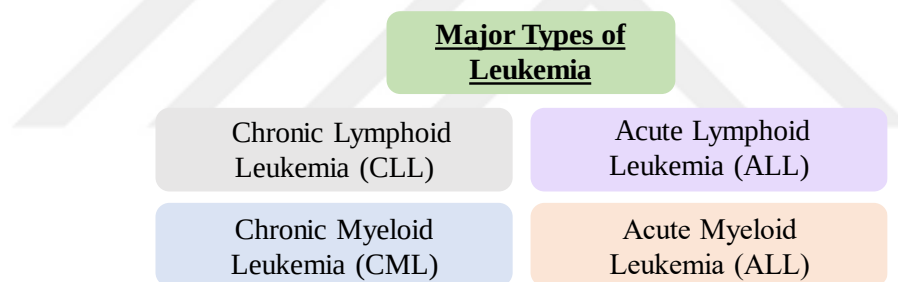


**Figure 1.6:** Major classification in leukemia

Chemotherapy, radiation therapy, and stem cell transplantation are the conventional treatment methodologies for leukemia patients. Although the median survival rate for children with Acute Lymphoid Leukemia (ALL) is close to 90%, this rate remains at 60% in children with Acute Myeloid Leukemia (AML) [American Cancer Society, 2020]. Therefore, new and effective therapy methods are being investigated.

### 1.2.1 Acute Myeloid Leukemia

Acute myeloid leukemia is a type of leukemia that occurs with uncontrolled growth of myeloid cells that cannot complete their maturation process. These cells are usually poorly differentiated during hematopoiesis. Although AML was incurable 50

years ago, it is now cured in 35 to 40% of adult patients who are younger than 60 years old [Dohner, 2010]. Cytogenetic heterogeneity in AML patients has been known for 30 years, and this character of the disease has become more evident in recent years [Medeiros et al., 2015]. Undoubtedly, targeted therapies for AML treatment with a better characterization at the molecular level is expected to be more effective.

During hematopoietic differentiation, megakaryocytes, erythrocytes, mast cells, and myoblasts develop from the myeloid origin [Gabrilovich et al., 2012]. While thrombocytes mature from megakaryocytes; basophils, neutrophils, eosinophils, and monocytes mature from myeloblasts. Some patients with AML have a subtype called myeloid leukemia, which usually affects neutrophils, some patients have another type of AML called monoblastic or monocytic leukemia, which usually affects monocytes. Less frequently, AML has another type called megakaryocytic, which grows from erythroid or platelets [Jagannathan-Bogdan and Zon, 2013].

The World Health Organization classifies different AML's according to cell forms, genetic disorders, dependence on therapy (therapy-related myeloid neoplasm), and their emergence due to other diseases [Arber et al., 2016]. In this study, treatment modalities are recommended for chromosomal translocated AML patients.

### 1.2.2 *Chromosomal Translocations in Acute Myeloid Leukemia*

Genomic stability is essential for the regular functioning of healthy cells, and cancer cells are generally known for their genomic instability [Yao and Dai, 2014]. Cytogenic changes causing deletions, inversions, and translocations. While deletions cause loss of tumor suppressor genes, inversions and translocations cause activation of proto-oncogenes and formation of tumor-specific fusion proteins. If breaks occur within the genes on each chromosome involved, this situation creates a fusion gene that encodes a chimeric protein, which may drive tumorigenesis especially when the fusion gene encodes transcription factors leading to cell division [Rabbitts, 1994]. Not surprisingly, gene fusions formed as a result of chromosomal translocations are thought to be responsible for the onset of carcinogenesis in 20% of human cancers [Mitelman et al., 2007].

Balanced chromosomal translocations, which is the breakage of a chromosome followed by rejoining to another location, is a phenomenon that occurs in 30% of AML

patients. Therefore, they have been an important era of research due to their significance in recognizing genes involved in leukemogenesis and their direct relationship to the treatment of patients [Mrozek and Bloomfield, 2008].

There are 4 different groups of translocations in AML. The first translocation targets the Core-binding factor (CBF) gene. Genes in the CBF group function by forming complexes with other genes involved in the differentiation of several tissues. Alfa subunit of CBF functions by connecting to DNA and beta subunit of CBF increases transcriptional activity without binding [Liu et al., 1993]. AML1-ETO fusion occurs as a result of a translocation between the (8;21) chromosomes. Thus, the AML1 gene binds to CBF, initiating the transcription of target genes. In another CBF fusion, whose function changes as a result of the inversion-inv (16) of the 16th chromosome, CBF beta subunit creates CBFB-MYH11 fusion protein [Kundu and Liu, 2001]. Both translocations, despite the impairment in hematopoiesis, are not sufficient to initiate AML alone. AML1-ETO and CBFB-MYH11 translocations are found to be present in approximately 2-12% of cases with AML, and both translocations have been associated with good prognosis [Appelbaum et al., 2006].

The second group is the translocation of the Retinoic acid receptor alpha (RARA) gene (on chromosome 17) with the Promyelocytic leukemia (PML) gene (on chromosome 15).[Chen and Chen, 1992]. The RARA/PML fusion gene is found in 95% of cases with Acute Promyelocytic Leukemia (APL), suggesting that this fusion might be a driver change in APL tumorigenesis. [Medscape, 2020]. Another particular example is the fusion of RARA with the Promyelocytic Leukemia Zinc Finger (PLZF) gene (between chromosomes 11;17) [Rohr et al., 2012]. In both cases, the differentiation is delayed in the pre-myelocytic phase. Interestingly, All-trans retinoic acid (ATRA) increases healthy differentiation in APL patients [Fang et al., 2002].

The third group of translocations contains homeotic genes such as HOX. The expression of HOX genes is very important during spinal cord development, which is reported to be increased during hematopoiesis [Argiropoulos and Humphries, 2007]. The HOX genes regulate hematopoiesis by maintaining proliferation and differentiation. Translocation of the HOX genes is shown to interfere with normal hematopoiesis [De Braekeleer et al., 2014]. The fusion of HOX genes with Nuclear Pore Complex (NUP98) has been reported for several of the genes present in the HOX gene cluster (A9, A11, A13, C11, C13, D11, and D13) [Atlas Genetics Oncology, 2020]. While recent studies

have shown evidence for the impairment of the HOX gene translocations with the function of hematopoietic progenitors, these translocations alone are not enough to start leukemogenesis [Sitwala et al., 2008].

The translocations of the MLL gene constitute the fourth major type of rearrangement observed in leukemias. Mixed-lineage leukemia 1 (MLL1) or Lysine [K]-specific Methyl Transferase 2A (KMT2A) gene on chromosome 11q23 is disrupted in a unique group of AMLs and ALLs [Winters and Bernt, 2017], which occurs in ~10% of leukemia patients. Despite the development of new treatment modalities, MLL-rearranged leukemia patients still display poor survival rates [Steinhilber and Marschalek, 2018]. Detailed information on the biological roles of MLL and its fusion partners including MLL-AF9, will be discussed in the following sections.

### **1.3 *MLL-Rearranged Leukemias***

#### **1.3.1 *MLL1***

The MLL1 gene encodes a multiplex protein that is essential in the regulation of early development and hematopoiesis. MLL protein contains multiple conserved domains, one of which is responsible for Histone 3 lysine 4 (H3K4) methyltransferase activity. MLL mediates chromatin modifications, which usually lead to increased transcriptional activity [Winters and Bernt, 2017]. Interestingly, while the overexpression of the MLL gene results in increased methylation, its disruption has no effect on global methylation, which is probably due to increased activity in other H4K4 methylases [Mishra et al., 2014]. Despite the redundant methylases, homozygous deletion of the MLL gene is lethal and the heterozygous mutant embryos cannot produce enough cells for several hematopoietic lineages [Winters and Bernt, 2017; Yu et al., 1995].

The wild-type MLL1 gene is localized at 11q23 locus; its product is a 500 kDa protein and is expressed in a variety of normal human tissues [Tkachuk et al., 1992]. MLL is known to be involved in epigenetic regulation of several developmental genes such as HOXA9, HOXA7, HOXC8 [Chen and Armstrong, 2015].

The N- and C- terminal ends of the protein harbor different tasks, and thus gain importance in determining the mechanism that will occur due to translocation. The protein has many defined domains; MBD, Menin-binding domain; SNL, speckled nuclear localization domains; AT, AT hooks; RD, repression domains; BCR, breakpoint cluster

region; BD, bromodomain; PHD, PHD fingers. CS1 and CS2 are the caspase-1 cleavage sites, TAD, transactivation domain; SET, H3K4 histone methyltransferase domain [Winters and Bernt, 2017].

The amino terminal of the protein contains a binding domain for Menin. SNL domain, AT hooks, and repression domains, are also located at the N-terminus [Tkachuk et al., 1992; Zeleznik-Le et al., 1994; Ma et al., 1993]. The repression domain contains CxxC, this domain has homology to DNA methyltransferase 1 (DNMT1), which binds non-methylated CpG islands [Birke et al.2002].

The carboxyl-terminal of the protein contains a SET domain, which transfers methyl groups, and a transactivation domain, which contains binding sites for other transcription coregulators [Milne et al., 2002; Korkmaz et al., 2012].

Between the amino and carboxyl-terminal of the MLL protein, there are bromodomain, which recognizes acetylated lysine residues, and a plant homeodomain finger (PHD), which mediates protein interactions. The MLL protein is post-translationally cleaved by the caspase enzyme, leaving an N terminal fragment that does not contain the SET domain and TA domain [Hsieh et al., 2003]. Usually, MLL fusion proteins lose their carboxyl-terminal and mid-domains thereby their SET domain, TA domain, bromodomain, and PHD finger may be missing [Santillan et al., 2006; Winters and Bernt, 2017]. For a proper chromatin regulation, MLL-N and MLL-C domains must interact with each other and MLL must successfully associate with other regulatory complexes. These complexes include histone acetyltransferases (CBP/p300) and hMOF, which have recently been shown to be essential for MLL1 target gene expression [Ernst et al. 2001; Valerio et al., 2017].

The SET domain is dispensable for MLL, but Retinoblastoma-binding protein 5 (RbBP5)-Set1/Ash2 Histone methyltransferase complex subunit (Ash2L) and WD repeat-containing protein 5 (WDR5) interactions are required for H3K4 methyltransferase activity [Mishra et al., 2014; Cao et al., 2010].

### 1.3.2 MLL-AF9 Fusions

10% of leukemia cases have been reported to harbor an MLL fusion. The MLL gene has ~80 known translocation partners, but only 6 of these often appear in patient-related cases [Winters and Bernt, 2017]. ALL1-fused gene from chromosome 4, 9, 10 (AF4, AF9,

AF10), elongation factor for RNA polymerase II (ELL), eleven-nineteen leukemia (ENL) and Partial tandem duplication (PTD) are the conventional fusion partners of MLL1 gene (**Table 1.1**). While MLL-AF4 fusion is commonly seen in ALL patients, MLL-AF9 fusion is mostly seen in AML patients. Among these, 60% of ALL patients bearing a fusion protein have an MLL-AF4 translocation, and 30% of AML patients with a rearrangement carry an MLL-AF9 oncogene [Marschalek, 2016]. The presence of t (4; 11) (q21; q23), which is one of the hallmarks of infant ALL, results in the production of excessive WBCs and leads to extremely poor prognosis [Uckun et al., 1998]. Pediatric MLL-AF9 fusion results in poorer prognosis when compared to AML in general because a considerable number of young patients with this cytogenetic abnormality relapse and die [Zwaan et al., 2015]. MLL-AF9 leukemia has been shown to follow a leukemia stem cell model (LSCM) [Zhu et al. 2016]. Leukemia stem cells are shown to be initiated by MLL-AF9 overexpression [Krivtsov et.al 2006]. Mice LSCs can be produced, using a mouse model of human acute myeloid leukemia induced by MLL-AF9 oncogene [Somervaille and Cleary, 2006]. A reversible mouse model for MLL-AF9 driven AML shows the expression of highly invasive epithelial-mesenchymal transition (EMT)-related genes, which is associated with poor outcome [Stavropoulou et al., 2016]. Human MLL-AF9 overexpression also induces hematopoietic expansion in zebrafish [Tan et al., 2018].

**Table 1.1:** Diagnostic fusion gene detection frequencies in ALL and AML's prognosis.

| Leukemia Partners | <u>ALL</u> | <u>AML</u> |
|-------------------|------------|------------|
| AF4               | 60%        | 10%        |
| AF9               | 12%        | 30%        |
| AF10              | 3%         | 15%        |
| ELL               | <1%        | 11%        |
| ENL               | 18%        | 19%        |
| PTD               | <1%        | 11%        |
| Others            | ~5%        | 4%         |

Percentages are adapted from the published data by Marschalek, 2016.

### **1.4 Molecular and Epigenetic Mechanisms of MLL-AF9 Fusions**

Defects in epigenetic pathways involving increased expression levels or abnormal patterns of activity are one of the key drivers of cell proliferation in cancer. Epigenetic dysfunction has a major role in myeloid malignancy. Chromosomal translocations are known to generate novel oncoproteins in myeloid leukemia, and to induce epigenetic dysfunctions [Harris et al., 2012].

MLL-AF9 translocation causes epigenetic regulations that sustain the oncogenic potential of leukemia stem cells. Translocation at t (9; 11) (p22; q23) induces expression of self-renewal-associated genes such as MLL-AF9 target genes: homeobox A9 (HOXA9) and Meis homeobox 1 (MEIS1) [Zhu et al. 2016]. MLL is required for normal development as a major regulator. Therefore, as a result of MLL rearrangements, recruitment of MLL fusion partners which disturb the expression of MLL target genes. Thus, MLL provides a useful model for studying the link between epigenetic cell regulation and cancer development.

As a result of MLL-AF9 translocations, the SET domain -thereby the methyltransferase activity- of the MLL gene is lost, but rather other chromatin-modifying enzymes are recruited to the DNA target sequences via the AF9 fusion domain, disrupting the normal progression of hematopoiesis. In this impaired mechanism, overexpressing enzymes have attracted the attention of the researchers. DOT1L methyltransferase has been reported to be responsible for increased H3K79 methylation at HOXA9 and Meis loci in MLL-rearrangements. Interestingly, inhibiting DOT1L in leukemia bearing these rearrangements has been reported to induce cell death [Bernt et al., 2011; Chang et al., 2010; Daigle et al. 2011]. Furthermore, Bromodomain-containing 4 (BRD4) is overexpressed in MLL-AF9 leukemias, and either short-hairpin RNA (shRNA) or pharmacological inhibition of BRD4 result with anti-leukemic effect in cells and *in vivo* [Zuber et al., 2011]. AF4 and AF9 fusion partners are present in the super elongation complex (SEC), which normally binds to RNA polymerase II complex and initiates transcriptional elongation. In MLL-rearranged leukemias, the SEC is engaged in the DNA-binding domain of MLL via the fusion partner. This leads to aberrant transcription of MLL target genes [Prange et al., 2017]. Mediator associated kinases such as cyclin-dependent kinases CDK8 and CDK19 do not regulate cell cycle progression but are involved in transcription [Fant and Taatjes, 2019]. For instance, during AML progression,

these kinases have been shown to induce higher cell survival and proliferation [Pelish et al., 2015].

As a result of the above-mentioned rearrangements, new interactions may form and initiate leukemogenesis. For example, in MLL-AF9 rearrangements, the fusion protein not only loses the methyltransferase activity (the SET domain is lost) but on the contrary recruits a demethylase, namely Lysine-specific demethylase (LSD1). LSD1, when present on the target sequences of the MLL gene, exerts the opposite gene expression changes driving leukemogenesis. Furthermore, LSD1 inhibition upregulates myeloid differentiation and reduces leukemic cell proliferation [Fang et al., 2017]. LSD1 is also known to be overexpressed in several types of leukemias. Therefore, it represents a novel epigenetic target for AML therapy [Pandey and Wang, 2019].

While agents that reduce methylation have been approved for their use in AML, there is a growing interest for other epigenetic targets such as HDACs for the treatment of leukemia. In AML, no mutations in the HDAC gene have been reported, nevertheless, abnormal oncogenic fusion proteins due to translocation, recruit several epigenetic regulators including HDACs [San Jose-Eneriz et al., 2019]. Altered HDACs expressions have been reported to result in tumor development due to their interactions with oncogenes and tumor suppressor genes [Ropero and Esteller, 2007].

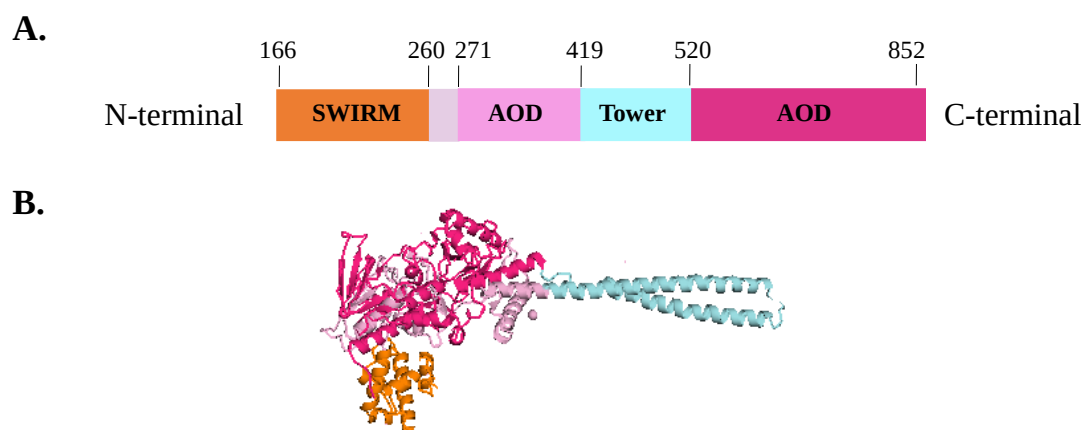
Therefore, in the context of this thesis, the contribution of LSD1 and HDAC enzymes to cancer development has been examined, and selective inhibition of these enzymes with pharmacological agents has been reviewed at the molecular level. Supporting evidence for the potential use of these enzymes as targets for the treatment of leukemia is reviewed in the following sections.

#### 1.4.1 *LSD1*

Lysine specific demethylase (LSD1), is the first identified flavin adenine dinucleotide (FAD)-dependent amine oxidase, which specifically removes one or two methyl groups from H3 lysine 4 and lysine 9 via a redox reaction [Chen et al., 2012]. The demethylation of H3K4 acts as a transcriptional co-repressor, while H3K9 acts as a transcriptional co-activator [Kozub et al., 2017]. The enzyme plays a major role in various biological processes including embryonic development, cell proliferation, EMT, DNA damage response, stem cell pluripotency, spermatogenesis and adipogenesis [Foster et

al., 2010; Majello et al., 2019; Ambrosio et al., 2017; Mosammaparast et al., 2013; Yin et al., 2014; Godmann et al., 2007; Musri et al., 2010].

The crystal structure of LSD1 was identified in 2006, two years after its discovery. LSD1 includes three structural domains: SWIRM domain on the N- terminal, FAD-binding amine oxidase domain (AOL) on the C- terminal, and a Tower domain located in between [Chen et al., 2006] (**Figure 1.7A**). SWIRM domain contains 85 amino acids and consists of mostly  $\alpha$ -helices, thus this domain is predicted to mediate protein interactions [Chen et al., 2012]. SWIRM and FAD domains are closely associated and form a spherical core through hydrophobic interactions [Kozub et al., 2017]. The Tower domain, which has a repeating pattern of seven residues, intervenes into the AOL and protrudes away from the AOL domain [Chen et al., 2012]. LSD1 enzyme, which does not contain a Tower domain (LSD1 $\Delta$ Tower) was reported to unable to reduce K4 methyl levels, supporting its essentiality for the demethylase activity of the enzyme [Chen et al., 2006]. LSD1 and co-repressor protein (CoREST) interactions are necessary for H3K4 demethylation [Yang et al., 2006]. On the other hand, H3K9 demethylation takes place through LSD1 complex with androgen or estrogen receptor (AR or ER) [Metzger et al., 2005; Carnesecchi et al., 2017]., LSD1 has an active role in different cell types and regulates several tissue-specific genes. Recently, a neuron-specific isoform of LSD1 (LSD1n) was described for the demethylation of H4K20me2, to activate neuron-regulated genes [Wang et al., 2015].



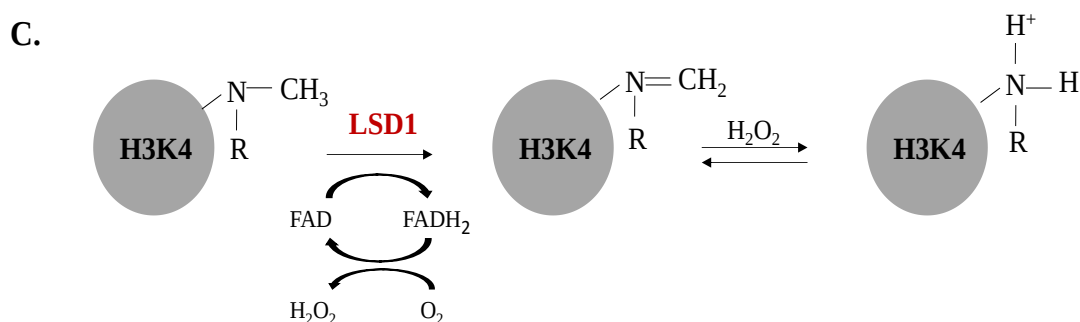


**Figure 1.7:** Structure of LSD1 enzyme and histone demethylation reaction

2D LSD1 domain structures (A). Human LSD1 enzyme in ribbon representation PDB accession code 2H94. The figure is created by using PyMol Software (B). The chemical reaction of the demethylation process performed by LSD1 enzyme (C), adapted from published data by Kozub et al., 2017.

The second member of the FAD-dependent demethylase family contains LSD2, which is a homolog of LSD1. These enzymes share sequence and structural similarity. While both contain AOL and SWIRM domains, LSD2 does not contain a Tower domain; instead, it contains an N-terminal zinc finger domain [Niwa and Umehara, 2017].

LSD1 is considered an oncogene and its overexpression may lead to uncontrolled cell proliferation, migration, and invasion [Kozub et al., 2017]. LSD1 dysregulation is related to human cancer development [Amente et al., 2013]. Methylation dynamics of several cancer-related proteins are also regulated by LSD1, including p53, E2F1, DNMT1, hypoxia-inducible factor-1 (HIF-1 $\alpha$ ), and Signal Transducer and Activator of Transcription 3 (STAT3) [Gu et al., 2020; Huang et al., 2007; He et al., 2018; Zhang et al., 2011; Lee et al., 2017; Yang et al., 2010]. Furthermore, LSD1 is reported to demethylate K370 residue of the protein which leads to repression of p53 [Huang et al., 2007].

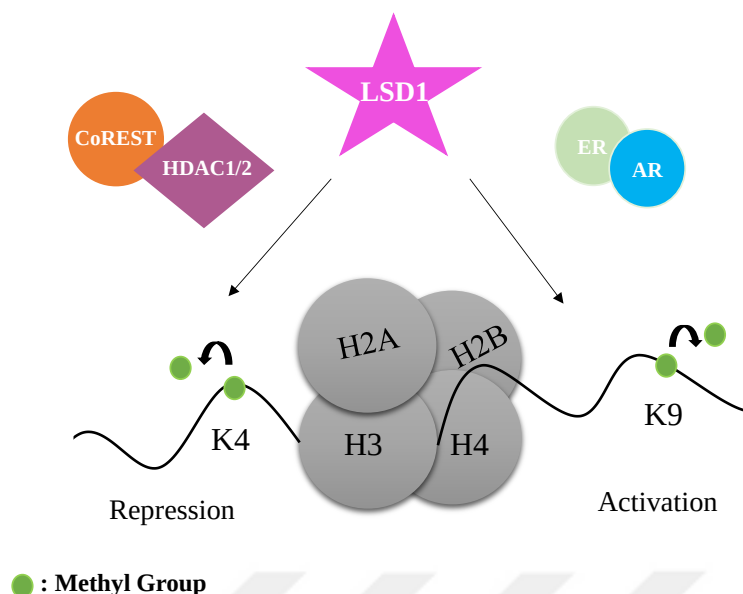


**Figure 1.8:** The role of LSD1 enzyme in transcriptional regulation

Represented data adapted from Kozub et al., 2017.

LSD1 is reported to repress the expression of cell migration-related genes through stabilization of the transcriptional regulator estrogen-related receptor  $\alpha$  (ERR $\alpha$ ) [Carnesecchi et al., 2017]. Similarly, LSD1 also plays a major role in AR-associated prostate cancer. Recently, it is shown that LSD1 induces the survival of castration-resistant prostate cancer by interacting with zinc finger ZNF217 [Sehrawat et al., 2017] and prevents autophagy by destabilizing p62 in gynecologic cancers [Chao et al., 2017].

Since LSD1 is an epigenetic regulator that controls various dynamics within the cell and plays an active role in many different types of cancer, LSD1 targeted drug discovery has attracted interest in the field of cancer research.

#### 1.4.2 LSD1 Inhibition in MLL-AF9 Leukemias

LSD1 plays a major role in normal hematopoiesis by interacting with growth factor independence (GFI) proteins. LSD1-CoREST-GFI complex mediates transcriptional repression for controlling hematopoietic differentiation. Uncontrolled activation of this complex results in an undifferentiated cell population [Saleque et al., 2007]. Concordantly, an inhibitor of this interaction, OG86, induced blast cell differentiation in acute myeloid leukemia (AML) [Maiques-Diaz et al. 2018].

LSD1 dysfunction and dysregulation are associated with both ALL and AML [Chen et al., 2012]. Inhibiting enzymatic activity of LSD1 resulted in decreased AML colony-

forming capacity in murine MLL-translocated AMLs [Harris et al., 2012]. The same study revealed that LSD1 knock-down downregulated MLL LSC maintenance, and the gene set enrichment analysis showed the downregulation of the oncogenic program. Chromatin immunoprecipitation (Ch-IP) analysis showed no global increase in H3K4me2 marks, but there was a retention of H3K4me2 marks specifically at MLL-AF9 target genes suggesting that LSD1 is responsible for the activation of MLL-AF9 oncogenes.

LSD1 is highly expressed in hematopoietic cells [Lynch et al., 2012] and the biological function of LSD1 is crucial. While LSD1 knock out in mice is embryonic lethal, LSD1, shRNA mediated knock-down induced the proliferation of hematopoietic stem and progenitor cells [Wang et al., 2007]. Thus, it is concluded as LSD1 overexpression sustains the LSC potential and is required for terminal differentiation of specific lineages [Mould et al., 2015]. Therefore, in the cases where LSD1 is overexpressed, known chemicals have been screened to determine compounds that decrease LSD1 activity and an antidepressant drug, tranylcypromine (TCP), identified to reduce LSD1 activity in a non-specific manner [Zheng et al., 2016]. TCP reduces the secondary LSC capacity for MLL-AF9 cells *ex vivo* [Harris et al., 2012]. Among the many different types of cancer lines, leukemic cell lines are more sensitive to LSD1 inhibition by TCP (**Figure 3.7**). Inhibition of LSD1 by TCP, reactivates the ATRA differentiation in AML, suggesting that TCP in combination with ATRA may target leukemia-initiating cells [Schenk et al., 2012]. Phase 1 studies have been started to evaluate the combinatorial effect of TCP and ATRA in AML patients. However, other TCP derivatives have been generated and investigated due to low specificity and low efficacy of the drug [Mould et al., 2015].

ORY-1001 is a patented (Oryzon Genomics) LSD1 inhibitor which induces differentiation and reduces leukemic stem cell activity in AML. The inhibitor reduces the growth of the AML xenograft model and increases the survival rate in a PDX model of T-cell acute leukemia [Maes et al., 2018]. ORY-1001 has a half-maximal inhibitory concentration (IC<sub>50</sub>) of 20 nM and is selective for LSD1 over other related targets such as LSD2 [Hojfeldt et al., 2013].

The compounds belonging to the class of Polyamines have also been identified as LSD1 inhibitors [Huang et al., 2007; Huang et al., 2009]. F14512, a polyamine-vectorized anti-cancer drug shows anti-leukemic activity, in combination with cytosine arabinoside (C-Ara), doxorubicin, gemcitabine, bortezomib, and SAHA. F14512 molecule also shows anti-leukemic activity when combined with Ara-C *in vivo* [Kruczynski et al.,

2013]. However, the lack of target selectivity is considered as a disadvantage for the polyamine groups.

A GlaxoSmithKline molecule, GSK2879552, is a selective, irreversible inactivator of LSD1 and indirectly, it also reduces the corepressor for element-1-silencing transcription factor CoREST activity by preventing them from binding together [Mohammad et al., 2015]. Inhibition of LSD1 by GSK2879552 activates the expression of tumor suppressor genes [Maes et al., 2015], enhances H3K4 methylation levels, exerts antiproliferative effects in AML cell lines [Mohammad et al., 2015; Zheng et al., 2016] and has recently entered Phase 1 clinical trials. In addition to AML treatment, GSK2879552 is the first LSD1 inhibitor that is recommended to be tested on solid cancers [Mould et al., 2015]. LSD1 inhibition with GSK2879552 reduces cell growth of many different AML cell lines with different translocations including MLL-AF4 and MLL-AF9. Also, macrophage marker expressions increase after treatment with GSK2879552 in MLL-AF9 fused MOLM-13 cell line. Additionally, a combination of GSK2879552 and ATRA enhances growth inhibition of AML cell lines and promotes caspase-dependent cell death; furthermore, reduces the colony-forming ability in patient-derived samples [Smitheman et al., 2019].

Multiple studies further characterized the effects of LSD1 inhibition *in vivo*. MLL-AF9 and green fluorescent protein (GFP) encoding retrovirus transduced with mouse hematopoietic progenitor cells, results in the emergence of AML in mice. Transplanted AML cells treated with GSK2879552 for 17 days shows 2.8% GFP positive cells, while the control has 80% GFP positive cells, suggesting that pharmacological inhibition of LSD1 reduces MLL-AF9 dependent tumor growth *in vivo* [Kruger et al., 2013].

SP2509 is a reversible inhibitor of LSD1, which attenuates the binding of LSD1 to the CoREST complex. SP2509 treatment reduces colony growth and induces apoptosis in MLL-AF4 and MLL-AF9 fused AML cell lines. SP2509 treatment mimics LSD1 knockdown in which induces cell differentiation. Furthermore, the combination of SP2509 and Panobinostat (a pan-HDAC inhibitor) shows anti-leukemic activity against AML xenografts [Fiskus et al., 2014]. In another study, two MLL-AF9 fused cell lines THP-1 and MOLM-13 show severe cell death which includes caspase 3/7 activation upon SP2509 treatment [Sonnemann et al., 2018]. However, SP2509, causes cell death on LSD1 knockout HAP1 cell line, suggesting the cell-killing potential of SP-2509 in AML cells is dominated by the off-target effects.

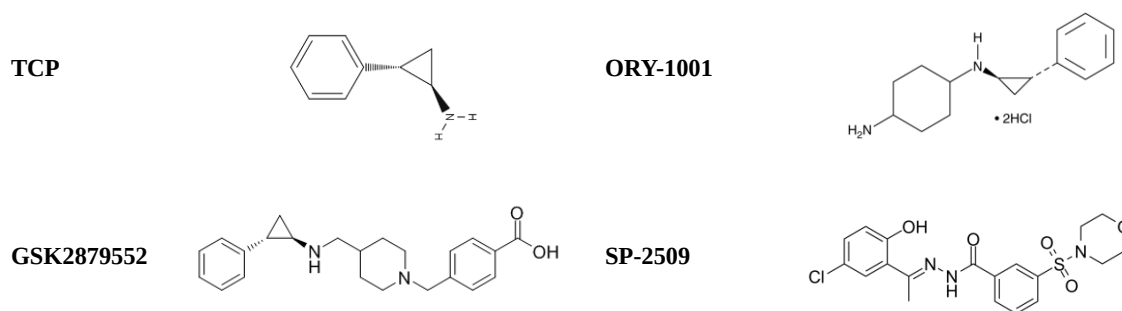


**Figure 1.9:** Chemical structures of LSD1 inhibitors

Since LSD1 is responsible for many biological pathways promoting tumor cell development, it represents an attractive molecular target for hematological malignancies, specifically in AML. Therefore, the discovery and synthesis of novel and specific LSD1 inhibitors are important for the availability of new and possible treatment modalities for AML patients.

#### 1.4.3 HDACs

Removal of acetyl groups from histones is catalyzed by Histone Deacetylases (HDACs) and they are classified based on their subcellular localization (i.e. nucleus, cytoplasm) and sequence homology (**Figure 1.3B**). While Class I, II, IV HDACs use Zinc as a cofactor for their catalyzes, Class III HDACs require  $\text{NAD}^+$  as a cofactor [Imai et al., 2000].

HDAC6 is reported to increase the deacetylation of histones *in vitro*, yet there is no report showing its interaction with histones *in vivo* [Grozinger et al., 1999]. Among the 18 mammalian HDACs, HDAC6 is a unique member, as most of the HDAC constitutive knockout mice are embryonic lethal or die within a few weeks after birth [Zhang et al., 2008].

HDAC6 is mainly localized in the cytoplasm due to nuclear exclusion (NES) and cytoplasmic anchoring serine/glutamate-rich repeat (SE14) motifs [Boyault et al., 2007]. HDAC6 contains NES domain adjacent to the N-terminal, following two catalytic domains (CD1 and CD2) [Ding et al., 2008]. Between these catalytic domains, HDAC6 contains a dynein motor binding (DMB) domain and following CD2, there is SE14 motif. The C-terminus of HDAC6 includes a zinc-finger ubiquitin-binding domain (ZnF) [Hai

and Christianson], which recognizes misfolded cargo proteins and these proteins are transported to aggresomes via dynein motors, on the other hand, CD1 introduces E3 ubiquitin ligase activity [Kawaguchi et al., 2003; Zhang et al., 2014]. Finally, the CD2 domain is the tubulin deacetylase domain and several selective HDAC6 inhibitors have focused specifically on this domain [Osco and Christianson, 2019].

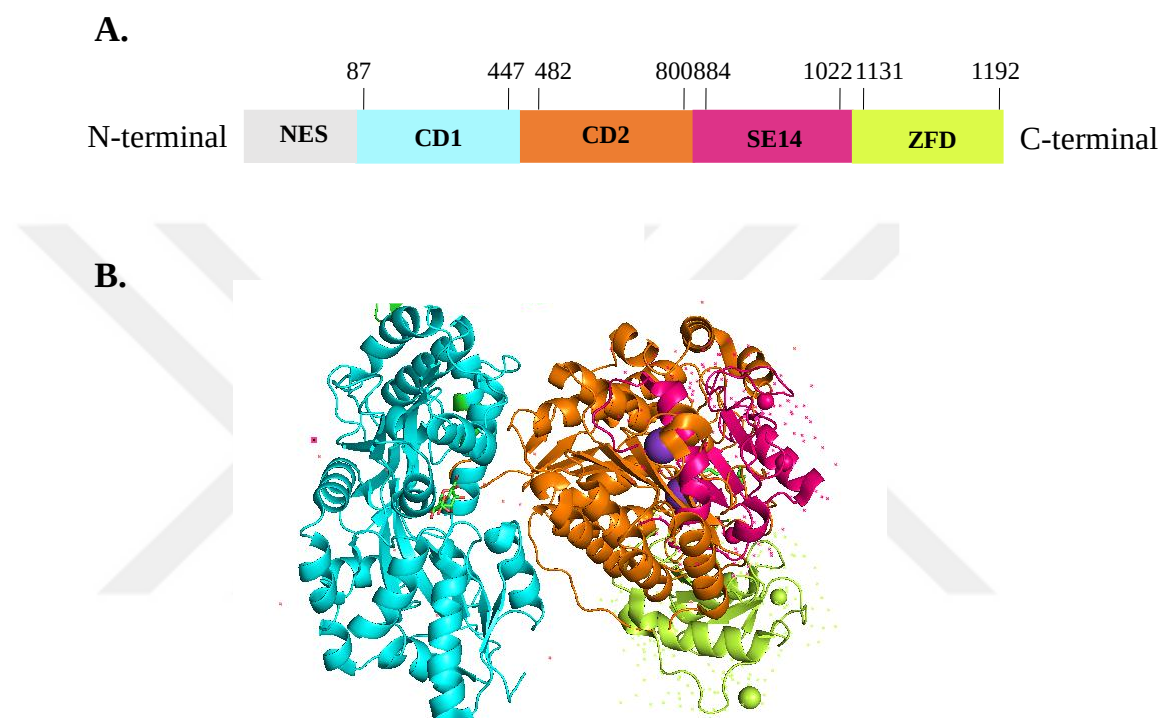


**Figure 1.10:** Structure of human HDAC6 enzyme

2D HDAC6 domain structures (**A**). Human HDAC6 enzyme in ribbon representation PDB accession code 3C5K, 3GV4, 5EDU. The figure is created by using PyMol Software (**B**).

The first identified cytoplasmic target of HDAC6 is  $\alpha$ -tubulin. Reversible acetylation of  $\alpha$ -tubulin is associated with microtubule stability and function [Piperno et al., 1987].  $\alpha$ -tubulin acetylation leads to microtubule stabilization and these acetyl groups are removed from  $\alpha$ -tubulins during dynamic cellular processes [Hubbert et al., 2002]. HDAC6 removes the acetyl group from the lysine 40 of tubulin subunit on the microtubules by the SE14 motif [Bertos et al., 2004]. Overexpression of HDAC6 induces tubulin deacetylation and results in increased cell motility [Szyk et al., 2014].

The second identified cytoplasmic target of HDAC6 is cortactin, another microtubule-dependent protein. Cortactin interacts with F-actin to promote branching

[Zhang et al., 2007] and is commonly seen at the leading edges of the migrating cells [Weaver et al., 2001]. Cell migration is increased upon cortactin overexpression [Kowalski et al., 2005; Patel et al., 1998], and impaired upon cortactin ablation [Bryce et al., 2005]. Acetylation of cortactin weakens its interaction with F-actin [Zhang et al., 2007], thus leading inhibition of cell mobility.

The third cytoplasmic target of HDAC6 is a heat shock protein 90 (HSP90). HSP90 is a chaperone protein that assists in protein stabilization, degradation, and proper folding [Jackson, 2013]. The stabilization of several proteins such as signal kinases, telomerase is also provided by HSP90 [Miyata et al., 2013]. Also, HSP90 inhibitors are being tested for their potential to be anti-cancer agents [Li et al., 2009]. HDAC6 associates with HSP90 through ubiquitin-binding and deacetylase domains. Inactivation or knockdown of HDAC6 results in HSP90 hyperacetylation and impairs chaperone activity [Kovacs, et al., 2005].

HDAC6 is a major regulator, considering its targets and effects on cell mobility, and its dysregulation might be considered as an important issue that requires more attention in terms of cancer.

#### 1.4.4 HDAC6 Inhibition in Hematologic Malignancies

HDACs have central roles in cancer development or progression by deacetylating histone and non-histone proteins, which are involved in the regulation of cell cycle, apoptosis, DNA damage response, metastasis, and autophagy. Therefore, restoring normal expression of HDACs may reverse cancer initiation or progression [Li and Seto, 2016]. To date, many HDAC inhibitors have been synthesized in different structures, however, the majority of these inhibitors are not specific for a certain HDAC, thus they come along with target-independent toxicities. Therefore, the discovery of a novel selective HDAC inhibitor gained importance for cancer therapy.

HDAC inhibitors are divided into 4 groups: hydroxamic acids, cyclic peptides, aliphatic acids, and benzamides [Tan et al., 2010]. NAD<sup>+</sup> dependent class of HDACs (Sirtuins) have an easier basis for selective inhibition [Magimaidas et al., 2016], while this circumstance, does not apply on Zinc dependent HDACs.

Tubacin was the first synthesized HDAC6 specific inhibitor, which increases the acetylated levels of  $\alpha$ -tubulin [Haggarty et al., 2003]. Tubacin treatment inhibits

proliferation and induces apoptosis through JNK/SAP kinase-independent pathways in ALL cells. Tubacin treatment also results in the accumulation of polyubiquitinated proteins and the cell viability may be further reduced by combination with Vincristine [Aldana-Masangkay et al., 2011]. Tubacin decreases tubulin deacetylase activity, however, it does not inhibit histone deacetylase activity and no supporting data has been reported its regulation with cell cycle progression.

Ricolinostat (ACY-1215) is another important HDAC6 inhibitor, which increases protein aggregation and  $\alpha$ -tubulin acetylation [Li et al., 2018]. Ricolinostat and Carfilzomib treatments increase apoptosis in lymphoma cells, and also reduces tumor growth and increase the survival of leukemic mice [Dasmahapatra et al., 2014]. Ricolinostat is reported to be a well-tolerated and effective therapeutic modality when combined with Bortezomib and Dexamethasone in multiple myeloma patients [Vogl et al., 2017]. HDAC6 inhibition (Ricolinostat) along with BET protein inhibition (JQ-1), shows a synergistic effect on multiple myeloma [Carew et al., 2019].

Tubastatin A is a potent HDAC6 inhibitor showing higher selectivity over other HDACs (excluding HDAC8). Tubastatin A is reported to induce cell death in several cancers including breast cancer, glioblastoma multiforme, and pancreatic cancer [Yuan et al., 2017; Li et al., 2017; Wang et al., 2012], yet hematologic malignancy-related data has not been provided so far.

Two pan-HDAC inhibitors (ST13, ST34) and an HDAC6 inhibitor (ST80) show growth inhibitory effect on leukemia cell lines including AML, and an increase histone and  $\alpha$ -tubulin acetylation [Hackanson et al., 2012]. Trichostatin A is reported to induce apoptosis in AML cells [Yu et al., 2008], yet the ability of Trichostatin A to inhibit Class I and Class II HDACs is thought to increase toxicity.

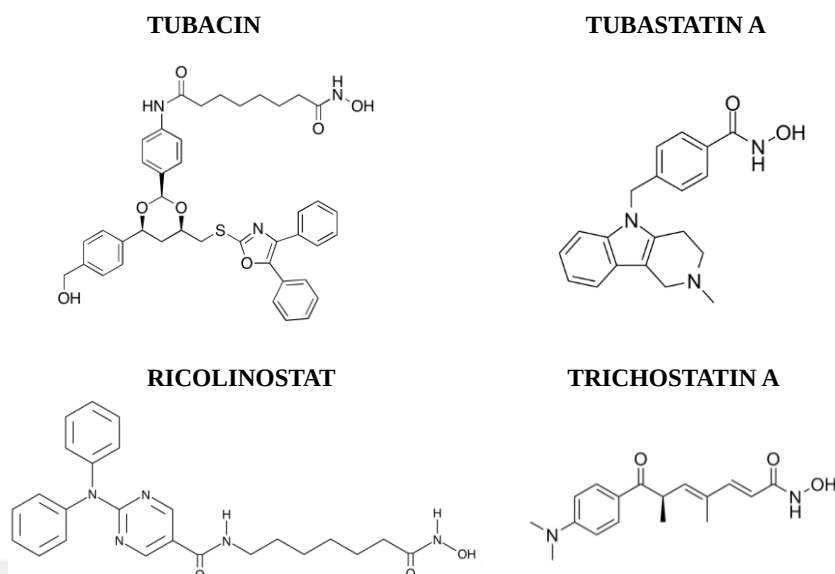


**Figure 1.11:** Chemical structures of HDAC6 inhibitors

HDAC inhibition has been reported to be effective against several types of cancers. On the other hand, HDAC6 appears to be a unique target in the context of cancer drug development. However, to date, the study of selective HDAC6 inhibition in AML cells has been limited due to the off-target effects of HDAC inhibitors. Therefore, synthesizing a selective HDAC6 inhibitor is needed in terms of leukemia research.

### 1.5 Chemotherapy Regimens in AML

Chemotherapy for AML usually involves a combination of at least 2 drugs. Conventionally, Cytarabine (CYT, Ara-C) is the first preferred drug and administered as a continuous infusion for 7 days. The majority of the patients then, receive an anti-tumor agent for 3 days. This chemotherapy approach in AML is referred to as “7-3 protocol”. There are four anti-tumor antibiotics: Daunorubicin (Cerubidine, daunomycin), Doxorubicin (Adriamycin), Idarubicin (Idamycin), and Mitoxantrone (Novantrone). In the context of this thesis, Doxorubicin and Cytarabine will be explained in more detail.

#### 1.5.1 Doxorubicin

Topoisomerase enzymes create temporary DNA strand breaks to allow relaxation of the coiled DNA [Kellner et al., 2002]. Topoisomerases mainly function in DNA replication, while they also have a role in chromosome segregation and condensation [Champoux, 2001; Durand-Dubief et al., 2010]. There are several FDA-approved

Topoisomerase II inhibitors including, Doxorubicin, Etoposide, Teniposide, and Mitoxantrone [Hande, 2003].

Doxorubicin (DOX) is a chemotherapy drug, which is an anthracycline, first extracted from *Streptomyces peucetius* var. *caesius* in 1969 [Arcamone et al., 1969]. The drug is routinely used in the treatment of several cancers including ALL, AML, multiple myeloma, breast, endometrial, gastric, head and neck, liver, kidney, neuroblastoma, ovarian and small cell lung cancer [Chemocare, 2020]. Cardiotoxicity is the major limitation for the use of Doxorubicin and pharmacokinetic studies are ongoing to reduce these side effects [Thorn et al., 2011].

There are two important pathways, where Doxorubicin is actively involved. Doxorubicin intercalates into DNA strands thus, inhibiting Topoisomerase II activity, and generates free radicals, which impair the structure of DNA and cellular proteins [Thorn et al., 2011]. Doxorubicin is reported to participate in the removal of histones from active chromatin, therefore DOX treatment alters transcriptome and attenuates DNA damage response, thus induces apoptosis in AML cells [Pang et al., 2013]. Cyclooxygenase-2 (COX-2) inhibitor Celecoxib with DOX combination has been shown to enhance growth inhibition and accumulation of G0/G1 phase cells in AML [Chen et al., 2013].

Long-term usage of high doses of Doxorubicin leads patients to develop drug resistance [Baran et al., 2007]. In this case, cells develop cross-resistance to the CYT as well. Therefore, in addition to known treatments, new treatment modalities to increase DOX's efficacy should be investigated.

### 1.5.2 Cytarabine

Cytarabine is one of the major chemotherapy drugs used in AML, ALL, and CML for more than 40 years [Lowenberg et al., 2011]. The drug is an arabinose sugar with a cytosine base, which is very similar to deoxy cytosine to be incorporated into human DNA, during the synthesis of the new strands. This similarity abrogates the replication, and the synthesis cannot be continued properly. The long-term S phase arrested cells go under programmed cell death due to the lack of synthesis of essential proteins [Li et al., 2017]. High-dose Cytarabine in AML treatment is a highly effective and frequently used therapeutic approach [Li et al., 2014]. However, in this model, patients suffer from side effects, therefore increasing the efficacy of Cytarabine and reducing the toxicity is an

important issue to overcome for a better clinical outcome. The combination of a small-molecule AZ20 and Cytarabine is reported to induce DNA replication stress and damage, following with AML cell death [Ma et al., 2017]. Another study reported that the addition of a lipophilic 5'-elaidic acid ester to Cytarabine structure increases the ability to overcome resistance mechanisms and offers an alternative to Cytarabine therapy [Rein and Rizzieri, 2014]. Several studies focus on the improvement of the Cytarabine transportation into the cell with higher efficacy, among these pro-drugs, amino acid conjugated ValCytarabine and fatty acid derivative CP-4055 are in the Phase III trials for the treatment of different types of leukemia [Chhikara and Parang, 2010].

The combination or improvement of many treatment modalities within the scope of AML with Cytarabine is being investigated despite the challenges. Considering the advantages and disadvantages, Cytarabine still has great importance in the treatment of leukemia.

### 1.5.3 Other Anti-Cancer Agents

There are more than 30 approved drugs for the treatment of AML [[National Cancer Institute, 2020](#)]. Amongst them, Paclitaxel, Cisplatin, and Etoposide are selected to assess their combinatorial cell-killing effects along with LSD1 and HDAC inhibition.

Paclitaxel (Taxol) is a member of the class of chemotherapy drugs called plant alkaloids and has been widely used for the treatment of several cancers [Rowinsky and Donehowe et al., 1995]. Taxol groups are referred to as microtubule poisons [Weaver et al., 2014] and the disruption of microtubule formation leads to failure in the depolymerization process resulting in the irreversible path of cell death [Rowinsky and Donehowe et al., 1995]. Paclitaxel is reported to have an anti-leukemic effect when combined with Cytarabine in AML cells [Curtis et al., 1996]. Leukemic cells are known to express a multidrug resistance gene (MDR1) [Lopez et al., 2001], thus they easily develop resistance to Taxol groups in long-term use. Therefore, epigenetic regulation, which reverts this resistance may result in sensitization of AML cells against Paclitaxel.

Cisplatin is characterized in 1845 and its anti-tumor activity discovered in the 1970s. Cisplatin cross-links DNA, forms adducts, and results in poorly repaired DNA damage, due to the accumulation of high-mobility-group proteins [Dasari and Tchounwou, 2014]. Cisplatin is approved by the FDA in 1978 for the treatment of testicular cancer and now

used in many cancers including ovarian, cervical, head and neck, esophageal, and non-small cell lung cancer [Jamieson and Lippard, 1999]. Several studies suggested using Cisplatin in multiple cancers, but the use of the drug remained limited in hematologic cancers. The combination of high-dose Cytarabine, Etoposide, and Cisplatin has been reported to reduce the growth of adult AML [Lee et al., 2014]. Another study revealed a sensitizer effect of Cisplatin to Temozolomide in leukemia cells and 25% of the patients demonstrated a reduction of blast cells in the bone marrow [Seiter K. et al., 2009]. Interestingly, one research revealed that a patient who diagnosed with AML and squamous cell carcinoma (laryngeal) was received Cisplatin chemoradiotherapy for his laryngeal cancer and simultaneously completely healed from AML [Mody et al., 2016]. The efficacy of Cisplatin in AML is not fully understood and further research on the mechanism of action is required.

Etoposide is synthesized in 1966 and approved for cancer therapy in 1983 by the FDA [Hande, 1998]. There are two Topoisomerase II in humans, TopoII $\alpha$  and TopoII $\beta$ . Both isoforms are active in the proliferation process, while TopoII $\beta$  also takes place in post-mitotic cells. TopoII $\alpha$  is known to function in cell cycle events such as DNA replication, whereas TopoII $\beta$  has been reported to take an active role in transcription [Lyu et al., 2006]. Etoposide is the inhibitor of both TopoII isomers, each inhibition results in cell death through different pathways. Early studies revealed a safe use of Etoposide as a single agent or along with Cytarabine and Azacitidine, resulting in remission rates up to 60% in patients with secondary AML [Ho et al., 1991]. However, a decade later, Etoposide is reported to induce a cleavage near leukemia-associated MLL translocation breakpoints [Lovett et al., 2001]. Now, Etoposide is known to induce secondary AML, through translocation between MLL and several partner genes including AF4 and AF9 [Montecucco et al., 2015; Ezoe et al., 2012]. In contrast, two clinical trials are investigating the efficacy of Etoposide along with Ciprofloxacin (NCT02773732) and Tipifarnib (NCT00112853) in AML patients. Despite the opposing views, Etoposide still has the potential of treating AML patients and further research on the mechanism of action is required.

### **1.6    *The Importance of Novel Epigenetic Therapies in AML***

Epigenetic dysregulations have been shown to play an important role in carcinogenesis [Kim et al., 2015]. However, their contributions to leukemogenesis are not fully elucidated [Shih et al., 2012]. Conventionally, AML is sensitive to treatments at the initial stage of the disease, however, due to the reproduction of resistant cells, it may relapse within the first few years. Especially for young patients, the recurrence of the disease significantly reduces the quality of life and survival rate [Gambacorta et al., 2019]. Therefore, alternative approaches and therapeutic agents are urgently needed for the treatment of AML.

Epigenetic regulations have a central role in cell differentiation together with the cell niche [Goldberg et al., 2007]. Overgrowth of LSCs during hematopoietic development may be eliminated by inhibition of several epigenetic regulators, thus preventing relapse [Sun et al., 2018]. Epigenetic reprogramming is known to reverse resistance in relapsing cancers including childhood B-lymphoblastic leukemia and multiple myeloma [Bhatla et al., 2012; Issa et al., 2017; Wilting and Dannenberg, 2012]. Therefore, epigenetic therapy may sensitize or re-sensitize cancer cells to conventional chemotherapies.

Multiple drugs that targeting epigenetic regulators have been approved by the FDA and many are still under investigation for AML therapy. In this context, Azacitidine and Decitabine are approved drugs targeting DNMT regulators [Gambacorta et al., 2019]. The 2-hydroxyglutarate, targeting TET enzymes, has recently started to be examined in clinical trials [Ye et al., 2018]. MM-401 and MI-503 are two small molecules targeting MLL-WDR5 and MLL-Menin complexes respectively, which are also under clinical investigation [Gambacorta et al., 2019]. Pinometostat is a DOT1L methyltransferase domain inhibitor of MLL that has recently passed clinical trials [Stein et al., 2018]. Similarly, clinical trials of NCD25 and GSK2879552 have successfully ended SP-2509 for the same target and are still in the studies. Finally, Panobinostat and Varinostat targeting class I/II HDACs was tested with clinical trials and approved for AML treatment [Gambacorta et al., 2019].

Conventional drug design generally comprises a “one drug-one target” strategy [Aggarwal, 2010]. Instead of intaking many single-targeted inhibitors and suffering from their individual side effects, the intervention of two- or more-targeted single-molecule became preferable in malignant cancers [Petrelli and Giordano, 2008]. In this context,

designing of multi-targeting drugs have attracted considerable attention for the past decade [Seo, 2015]. There are several studies revealed the superior potency of “dual targeting” inhibitors. Novel dual inhibitors targeting CDK4 and VEGFR2, reduce cancer progression, and angiogenesis compared to single inhibition [Huang et al., 2019]. Dual inhibition of Topoisomerase II and epidermal growth factor receptors (EGFR) shows anticancer activity at the micromolar level against a panel of cancer cells [Chauhan et al., 2016]. PIK-75 is reported to simultaneously inhibit Cdk9 and PI3K and shows a cytotoxic activity in AML cells [Thomas et al., 2013]. MEN1703 is a dual inhibitor of PIM and FLT3 kinase that overcomes FLT3 resistance in AML patients and reduces cell growth of AML regardless of FLT3 mutational status [Ravandi et al., 2019]. In addition, dual inhibitors of FLT3/PDGFR (Platelet-derived growth factor) (Crenolanib), FLT3/Mer (Mer receptor tyrosine kinase) (UNC2025), and FLT3/SYK (Spleen tyrosine kinase) (Midostaurin) are reported to have anti-leukemic activity and revert drug resistance in AML cells [Yuan et al., 2019]. Therefore, the sensitivity of AML cells against dual inhibitors may be leveraged for therapeutic benefit.

In this thesis, *in vitro* enzymatic and the biological activities of novel inhibitors of LSD1(AD-94), HDAC6 (MH-25), and a dual inhibitor which targets both LSD1 and HDAC6 (AD-58) are investigated on different AML cell types. A chemically altered isomer (MH-18) and inhibitors under clinical investigation (GSK2879552 and Ricolinostat) are used as negative and positive controls, respectively. The efficacy of combinatorial treatment of the inhibitors with selected clinical agents for MLL-rearranged AML's is also evaluated and finally, neoteric treatment modalities are proposed for MLL-AF9 leukemias.

## Chapter 2:

### MATERIALS AND METHODS

#### 2.1 Design and Synthesis of Novel LSD1/HDAC6/Dual Inhibitors

*“The design and synthesis of mentioned inhibitors belong to the hard work of Prof. Dr. Arasu Ganesan and Dr. Adam Lee from the School of Pharmacy Department of East Anglia University.”*

AD-58/iDual was derived from the reference molecule GSK2879552 following the described procedures in (International Patent Publication Number: WO 2012/135113 A2) except the methyl benzoate (1) was used in place of the tert-butyl benzoate. This allowed conversion of the methyl-ester (5) precursor of GSK2879552, to AD-58/iDual in one step.

Both MH-18/iNeg.Cntrl and MH-25/iHDAC6 were synthesized using the same reaction as for GSK2879552 and AD-58/iDual, with the exception that 1-phenyl-1-cyclopropylamine was used in place of tranlylcypromine (TCP) (**Figure 2.1**).

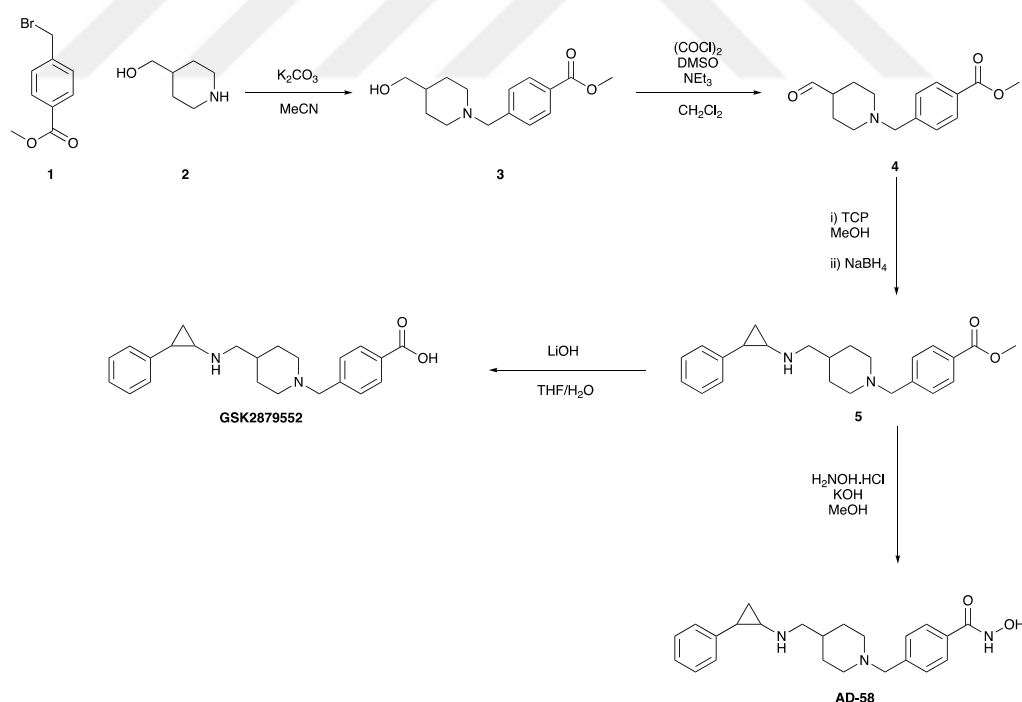


**Figure 2.1:** Synthesis reaction of AD-58 dual inhibitor

The figure is adapted from Lee et al. University of East Anglia, 2020 (unpublished data)

AD-94/iLSD1 was developed based on the structure of the precursor molecule GSK2879552 as a novel LSD1 inhibitor.

Following synthesis of GSK2879552, a single step reaction via amine coupling between GSK2879552 and 1-(3-aminopropyl) imidazole using EDC and a catalytic quantity of HOBT led to the formation of AD-94 (**Figure 2.2**).

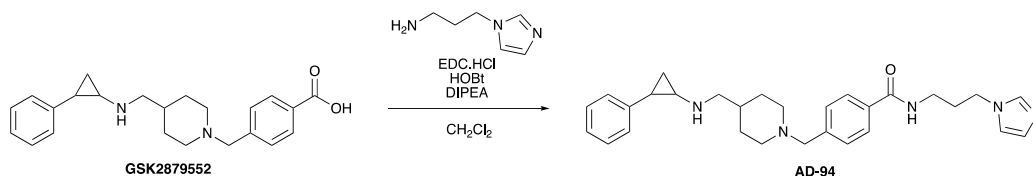


**Figure 2.2:** Synthesis reaction of the AD-94 LSD1 inhibitor

The figure is adapted from Lee et al. University of East Anglia, 2020 (unpublished data)

## 2.2 Cell Lines and Reagents

THP-1, Jurkat, and K-562 cell lines were purchased from ATCC (TIB-202, CCL-243, TTIB-152) and the MOLM-13 cell line was purchased from DSMZ (ACC-554). All cell lines were routinely grown at 37 °C in a 5% CO<sub>2</sub> humidified atmosphere in RPMI-1640 (Sigma Aldrich #R8758) supplemented with heat-inactivated 10% fetal bovine serum (FBS; Biowest #1810), 100 U/ml of penicillin, and 100 µg/ml of streptomycin (Life Technologies #15140122).

## 2.3 Measuring LSD1 Activity by In-vitro Enzyme Assays

LSD1 (KDM1A) Fluorescent Assay Kit (BPS Bioscience #50106) protocols were followed to measure LSD1 enzyme activity. Briefly, a mix including methylated peptide and LSD1 Assay Buffer was prepared and added in a black 96-well plate. Different dilutions of the test inhibitors were added to the mix and incubated with LSD1 enzyme (10ng/µl) for 30 minutes at room temperature. After incubation, 10 mM Amplex Red and 10 U/ml Peroxidase mix were added to the wells. The plate was incubated at room temperature for 5 minutes. The fluorescence signal was detected at  $\lambda_{ex}$ =530 nm and  $\lambda_{em}$ =590 nm with Bio-Tek H1 Synergy microplate reader. Blank value was subtracted from all measurements.

## 2.4 Measuring the Activity of Different HDACs via In-vitro Enzyme Assays

*“Measuring the HDACs activities belong to the hard work of Dusan Ruzic, Katarina Nikolic, and Sheraz Gul.”*

In order to measure HDAC 1 and 3 activity, HDAC Glo I/II Assay (Promega, SOP-IME-SP-032) was used following the manufacturer's protocols. The assay is based on the cleavage of luciferin labeled peptide by active HDACs. Cleaved amino luciferin emanates luminescence upon cleavage via luciferase enzyme depending on the HDACs activity. For this, the cleaved peptide was progressed by Developer Reagent. Compounds and controls were added in assay plate with via Echo Liquid handling system. 5  $\mu$ L Assay Buffer added to blank wells and 5  $\mu$ L enzyme solution added to the control and test wells. The plate was centrifuged at 1.000 rpm for 5 minutes and incubated at room temperature for 10 minutes. 5  $\mu$ L HDAC Glo I/II Reagents added to wells and the plate was centrifuged at 1.000 rpm for 5 minutes. The assay plate was incubated at room temperature for 10 minutes and the luminescence signal was detected on a multi-plate reader.

## 2.5 Cellular Thermal Shift Assay

THP-1 cells were seeded in T75 flasks with  $1 \times 10^6$ /mL and treated with the selected inhibitors for 1 hour at 37°C and 5% CO<sub>2</sub>. 10 ml of treated and untreated cells were centrifuged, and the pellet was resuspended with Phosphate Buffer Saline (Gibco #10010023) containing Protease Inhibitor Cocktail Pi (Cell Signaling Technology #5871) and 1 mM Phenyl methyl sulfonyl fluoride PMSF (Sigma Aldrich #10837091001). The cell suspension then was divided equally and heated for 3 minutes at 43, 46, 49, 52, 55, 58, 61, and 64°C on BIORAD T100 Thermal Cycler. Cells were snap-frozen and thawed twice and centrifuged at maximum speed (17.000 g) for 15 minutes at 4°C. The protein-containing supernatant was collected and loaded on SDS-PAGE gel (BIORAD #161-0317). After electrophoresis (100 V and 60 minutes) proteins were transferred onto PVDF membrane (Bio-Rad #1620177) (200mA for 180 minutes) and immunoblotted with primary antibodies (1:10.000 dilution for Anti-LSD1 Antibody ab190507, 1:1.000 dilution for Anti-HDAC6 Antibody CST 7558, 1:1.000 dilution for Anti-HDAC8 Antibody ab187139, 1:1.000 dilution for Anti-HDAC1 Antibody ab53091, 1:5.000 dilution for Anti-beta Actin Antibody ab8227) for overnight at 4°C. The next day,

membranes were incubated with an HRP-conjugated secondary antibody (1:2000 dilution for Abcam, ab205718) for 1 hour at room temperature. Chemiluminescent signal was detected by using Immobilon HRP Substrate (WBKLS0500) with BIORAD Chemi-Doc XRS+ System.

## **2.6 Measurement of LSD1 Expression in Different Cells Lines**

### **2.6.1 Cell Culture**

Leukemia cell lines (Jurkat, K-562, MOLM-13, THP-1) were grown in RPMI-1640 (Sigma Aldrich #R8758) supplemented with heat-inactivated 10% fetal bovine serum (FBS; Biowest #1810) and 100 U/ml of penicillin, and 100 µg/ml of streptomycin (Life Technologies #15140122). Breast cancer cell lines (SUM-149, MDA-231, MCF-7), brain tumor cell lines (U87-MG, U373, T-98, LN18, A172), Hek293T and HeLa cells were grown in DMEM (Sigma Aldrich #51434C) supplemented with heat-inactivated 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml of penicillin, and 100 µg/ml of streptomycin. Prostate cancer cell lines 22Rv1, PC-3, DU-145, LNCaP cell lines were grown in RPMI-1640 (Sigma Aldrich #R8758) supplemented with heat-inactivated 10% fetal bovine serum, 100 U/ml of penicillin, and 100 µg/ml of streptomycin. Non-small lung cancer cell lines (A-549, H1299), COV434 and Human Villous Trophoblasts (HVT) were grown in DMEM/F-12 (Gibco #11320033) supplemented with heat-inactivated 5% fetal bovine serum, 100 U/ml of penicillin, and 100 µg/ml of streptomycin.

### **2.6.2 Protein Extraction**

Protein extractions were performed by using RIPA Lysis Buffer (Sigma Aldrich, #R0278) containing 1mM PMSF (Sigma Aldrich #10837091001) and Protease Inhibitor Cocktail (Cell Signaling Technology #5871). Cells were harvested and centrifuged at 300 g 5 minutes. Cell pellets were resuspended with RIPA Lysis Buffer for 10 million cells/mL. The suspension was centrifuged at 17.000 g 20 minutes at 4°C. Protein containing supernatant kept on ice during experiments.

### 2.6.3 *Measuring Protein Concentration*

Pierce™ BCA Protein Assay Kit (Thermo Fischer Scientific #23225) was used to measure protein concentrations. 2mg/ml Bovine Serum Albumin standard diluted at different concentrations (0, 0.4, 0.8, 1.2, 1.6, 2 mg/ml) with ddH<sub>2</sub>O. Working solution mix, containing Reagent A and Reagent B (50:1 ratio) were prepared. BSA standards and proteins were added into 96 well plates (10 µL/well) and mixed with BCA working solution (190 µL/well). The plate incubated at 37 °C for 30 minutes. Absorbance was measured at 562 nm with Bio-Tek H1 Synergy microplate reader. Blank values were subtracted from all measurements. Protein concentrations were normalized to the standard curve.

### 2.6.4 *SDS-PAGE and Western Blotting*

30 µg of protein for each sample was loaded on 4-15% SDS-PAGE gel (Mini-PROTEAN® TGX™ Precast Gels, BIORAD #161-0317). After electrophoresis (100 V and 60 minutes) proteins were transferred onto the PVDF membrane (Bio-Rad #1620177) at 200 mA for 180 minutes and immunoblotted with primary antibody (1:10.000 dilution for Anti-LSD1 Antibody ab190507) for overnight at 4 °C. Next day membrane was incubated with HRP-conjugated secondary antibody (1:2.000 dilution for Abcam, ab205718) for 1 hour at room temperature. Chemiluminescent signal was detected by using Immobilon HRP Substrate (WBKLS0500) with BIORAD Chemi-Doc XRS+ System. Band intensities were quantified using ImageJ software.

## 2.7 *Cell Viability Tests upon Treatment with Tranilcypromine*

### 2.7.1 *Drug Treatment*

$2 \times 10^3$  cells (Hek293-T, MCF-7, 22Rv1, A-549, HeLa, DU-145, MDA-231, PC-3, SUM-149, and THP-1) were seeded in 96 well plates and treated with 2mM of Tranilcypromine (TCP) for 96 and 120 hours.

### 2.7.2 *Cell Viability Tests with SRB*

Cells were fixed with 50 µL of 50% Trichloroacetic acid (TCA, Sigma Aldrich, T6399) for 1 hour at 4 °C. Wells were washed with dH<sub>2</sub>O for 5 times and 50 µL % 0.4

(w/v) Sulforhodamine B (SRB) solution (Sigma Aldrich, 230162) was added and the plate was incubated for 30 minutes at room temperature. At the end of the staining period, the plate was washed with a 1% Acetic Acid Solution (Sigma Aldrich, 45754). To dissolve SRB, 150  $\mu$ L 10 mM Tris-Base solution was added to the wells, and the absorbance (562 nm) was detected with Bio-Tek H1 Synergy microplate reader.

## **2.8 Cell Viability Tests upon Treatment with Novel Inhibitors**

$2 \times 10^3$  cells were seeded in black 96 well plates and treated with indicated inhibitors (AD-94, AD-58, MH-25, MH-18, GSK2879552, and Ricolinostat) for 120 hours. Cell viability was measured with Promega Cell Titer-Glo® Luminescent Cell Viability Assay. Cell Titer-Glo Substrate (G755A) and Cell Titer-Glo Buffer (G756A) mixed in 1:1 ratio and added (20  $\mu$ L/well) to the wells. Plates were incubated at 37°C for 20 minutes. Luminescence signal was detected with Bio-Tek H1 Synergy microplate reader.

## **2.9 H3K4 and H3K9 methylation Levels upon LSD1 Inhibition**

### **2.9.1 Drug Treatment**

H3K4me2 protein levels were investigated upon treatment with LSD1 inhibitors on THP-1 and MOLM-13 cell lines after 24 hours.  $3 \times 10^5$  cells were seeded in 6 well plates and treated with 2  $\mu$ M of indicated inhibitors (Tranylcypromine, AD-94, GSK2879552, and AD-58).

### **2.9.2 Histone Extraction**

Cells were centrifuged at 300 g for 5 minutes. Cell pellets were resuspended with Triton Extraction Buffer containing 2 mM PMSF at a final concentration of  $1 \times 10^6$  cells/mL and incubated on ice for 10 minutes. Cell suspensions then, centrifuged at 6.500 g for 10 minutes at 4°C. The supernatant was discarded, and the pellet was resuspended with the same volume of TEB buffer and centrifuged at 6.500 g for 10 minutes. The pellet was resuspended with 0.2N HCl overnight at 4°C. The next day, samples were centrifuged, and histone protein-containing supernatant was normalized with a 1:4 volume of 0.1 M NaOH.

### 2.9.3 *Measuring Protein Concentration*

Measuring protein concentrations were performed as described in Chapter 2.6.3.

### 2.9.4 *SDS-PAGE Gel Electrophoresis and Western Blotting*

SDS-PAGE and western blotting were performed as described in Chapter 2.6.4 by using 2 µg histone proteins of each sample. Following the transfer, the membrane was incubated with primary antibodies (1:1.000 dilution for Abcam Anti-Histone H3 monomethyl K4 antibody ab8895, 1:1.000 dilution for Abcam Anti-Histone H3 dimethyl K4 antibody ab7766, 1:1.000 dilution for Abcam Anti-Histone H3 monomethyl K9 antibody ab9045, 1:1.000 dilution for Abcam Anti-Histone H3 antibody ab18521) for overnight at 4°C. The next day, the membrane was incubated with an HRP-conjugated secondary antibody (1:2.000 dilution for Abcam ab205718) for 1 hour at room temperature. The membrane imaging was performed as described in Chapter 2.6.4. Band intensities were quantified using ImageJ software

## **2.10 *Acetylated $\alpha$ -tubulin Levels upon HDAC6 Inhibition***

### 2.10.1 *Drug Treatment*

Acetylated  $\alpha$ -Tubulin protein levels were investigated upon treatment with HDAC6 inhibitors on THP-1 cell line for 24 hours.  $3 \times 10^5$  cells were seeded in 6 well plates and treated with 4 µM of indicated (MH-25, AD-58, Ricolinostat) inhibitors.

### 2.10.2 *Protein Extraction*

Protein extraction was performed as described in Chapter 2.6.2.

### 2.10.3 *Measuring Protein Concentration*

Measuring protein concentration was performed as described in Chapter 2.6.2.

### 2.10.4 *SDS-PAGE Gel Electrophoresis and Western Blotting*

SDS-PAGE and western blotting were performed as described in Chapter 2.6.4 by using 30 µg total proteins of each sample. Following the transfer, the membrane was

immunoblotted with primary antibodies (1:1.000 dilution for Cell Signaling Technology Acetyl- $\alpha$ -Tubulin (Lys40) (D20G3) XP® Rabbit mAb 5335, 1:1.000 dilution for Abcam Anti-alpha Tubulin antibody ab18251) for overnight at 4 °C. The next day, the membrane was incubated with an HRP-conjugated secondary antibody (1:2.000 dilution for Abcam, ab205718) for 1 hour at room temperature. The membrane imaging was performed as described in Chapter 2.6.4. Band intensities were quantified using ImageJ software.

## **2.11 Macrophage Marker Expressions upon LSD1 Inhibition**

### **2.11.1 Drug Treatment**

$3 \times 10^5$  THP-1 cells were seeded in 6 well plates and treated with 1  $\mu$ M of indicated inhibitors (AD-94, AD-58, MH-18, and GSK2879552) for 72 hours.

### **2.11.2 RNA Extraction**

Macherey Nagel™ NucleoSpin™ RNA Plus Isolation Kit (740955.50) protocols were followed for RNA isolation. Cells were centrifuged at 300 g for 5 minutes. Cell pellets were resuspended with RA1 Buffer containing  $\beta$ -mercaptoethanol, vortexed vigorously and centrifuged at 11.000 g for 1 minute. The supernatant was mixed with 70% ethanol and loaded on the RNA isolation column and centrifuged at 11.000 g for 1 minute. Columns were treated with 95  $\mu$ L rDNAse containing Reaction Buffer for 15 minutes at room temperature then, washed with RAW2 washing buffer twice and centrifuged at 11.000 g for 1 minute. RNAs were eluted with 50  $\mu$ L nuclease-free water.

### **2.11.3 cDNA Synthesis**

Thermo Fischer Scientific M-MLV Reverse Transcriptase (#28025013) was used for cDNA synthesis. Briefly, 500 ng of RNA was mixed with 10mM dNTP Mix and 10  $\mu$ M Oligo dT primer and incubated at 65 °C for 5 minutes. 4  $\mu$ L Reaction Buffer, 1  $\mu$ L 0.1 M DTT, and 1  $\mu$ L RNase inhibitor were mixed and added into RNA solution at 37 °C for 2 minutes. 1  $\mu$ L of M-MLV Reverse Transcriptase were added, and the solution was incubated at 37 °C for 60 minutes. Reverse Transcriptase enzyme was inactivated at 75 °C for 15 minutes and cDNA was kept on ice during the following experiments.

#### 2.11.4 RT-qPCR

Gene expression levels were quantified by SYBR Green qRT-PCR method using a LightCycler 480 SYBR Green I Master (Roche Diagnostics, #50-720-3180). Subsequently, 10 ng cDNA template was amplified using 10  $\mu$ L LightCycler 480 SYBR Green I Master (Roche Diagnostics), 3.5  $\mu$ L of nuclease-free water, and 2.5  $\mu$ L gene-specific primers mix in a final volume of 20  $\mu$ L. Quantitative RT-PCR was run on a LightCycler 480 System II (Roche Diagnostics). The reaction mixture was incubated at 95°C for 5 minutes, followed by 40 cycles of 95°C for 10 s, 55°C for 30 s, and 72°C for 1 s. The relative amounts of gene expressions were measured with the  $2^{(-\Delta\Delta CT)}$  method and normalized to GAPDH expression. All qRT-PCRs were performed in duplicates with three biological replicas.

**Table 2.1:** Macrophage marker genes and primer sequences.

| Gene  | Sequence (5'-3')           |
|-------|----------------------------|
| CD11b | F: TGTCAAGGGCACCCAGATCG    |
|       | R: AAGGGGCACACGGACACC      |
| CD86  | F: GGACTAGCACAGACACACGGAT  |
|       | R: AGCAGCACCAGAGAGCAGGAAG  |
| GAPDH | F: CTGACTTCAACAGCGACACC    |
|       | R: GTTGTTCATACCAGGAAATGAGC |

## 2.12 P21 Gene Expression Levels upon HDAC6 Inhibition

### 2.12.1 Drug Treatment

$3 \times 10^5$  THP-1 cells were seeded in 6 well plates and treated with 1  $\mu$ M of indicated inhibitors (AD-58, MH-25, MH-18, Ricolinostat) for 72 hours.

### 2.12.2 RNA Extraction

RNA extraction was performed as described in Chapter 2.11.2.

### 2.12.3 cDNA Synthesis

cDNA synthesis was performed as described in Chapter 2.11.3.

### 2.12.4 RT-qPCR

Gene expression levels were quantified with RT-qPCR analysis using the same protocols as described in Chapter 2.11.

**Table 2.2:** p21 specific RT-qPCR primers.

| Gene  | Sequence (5'-3')           |
|-------|----------------------------|
| P21   | F: TCGTCCAGCGACCTTCCTCA    |
|       | R: CAGCCCCATATGAGCCCACG    |
| GAPDH | F: CTGACTTCAACAGCGACACC    |
|       | R: GTTGTTCATACCAGGAAATGAGC |

## 2.13 Cell Viability Test with Combination Assays

### 2.13.1 Drug Treatment

$2 \times 10^3$  number of THP-1 cells were seeded in 96 well plates and treated with epigenetic inhibitors for 72 hours. Then cells were co-treated with commonly used anti-cancer agents (Cisplatin, Cytarabine, Paclitaxel, and Doxorubicin) for 48 hours.

### 2.13.2 Cell Viability Tests

Measuring cell viability was performed as described in Chapter 2.8.

## 2.14 Determination of Cell Death Mechanism upon Combination Therapy

### 2.14.1 Drug Treatment

$3 \times 10^5$  number of THP-1 cells were seeded in 6 well plates and treated with epigenetic inhibitors for 72 hours. Then cells were co-treated with Doxorubicin for 48 hours.

#### 2.14.2 Annexin-V Staining

Cells were centrifuged at 300 g for 5 minutes and the cell pellet was resuspended with 1% FBS in PBS. Cells and Annexin V dye (Millipore, MCH100105) were mixed in a 1:1 ratio and incubated at room temperature for 20 minutes, apoptotic cells were counted with Muse Cell Analyzer (Merck Millipore).

#### 2.14.3 Caspase 3/7 Staining

Cells were centrifuged at 300 g for 5 minutes and the cell pellet was resuspended with 1% FBS in PBS. The working solution is prepared (Caspase 3/7 reagent MCH4700-1505) mixed with PBS in a 1:8 ratio and mixed with 50  $\mu$ L of cell suspension and incubated for 30 minutes at 37°C. 7-AAD dye (MCH4700-1505) was diluted with Buffer BA (MCH4700-1360) in a ratio of 1:75. 150  $\mu$ L 7-AAD solution mixed with cell suspension and incubated for 5 minutes at room temperature. Apoptotic cells were counted with Muse Cell Analyzer (Merck Millipore).

#### 2.14.4 $\gamma$ H2AX Activation

Cells were centrifuged at 300 g for 5 minutes and the cell pellet was resuspended with Assay Buffer (CS202134). The cell suspension was mixed with the Fixation Buffer (1:2 ratio CS202122) and incubated on ice for 5 minutes. Cells were centrifuged and mixed with ice-cold Permeabilization Buffer (1:2 ratio CS203284) and incubated on ice for 5 minutes. Cells were centrifuged at 600 g and resuspended with 50  $\mu$ L of 20X Anti-H2A.X PECy5 (CS208202) and 20X Anti-phosphate (Ser139) Alexa Fluor 555 (CS208203) mix and incubated for 30 minutes at room temperature. Cells were centrifuged and resuspended with 200  $\mu$ L of 1X Assay Buffer.  $\gamma$ H2AX activated cells were counted with Muse Cell Analyzer (Merck Millipore).

#### 2.14.5 Reactive Oxygen Species Activation

Cells were centrifuged at 300 g for 5 minutes and the cell pellet was resuspended with 1X Assay Buffer (MCH100111-2). Oxidative Stress Reagent (4700-1665) was mixed with 1X Assay Buffer in a 1:100 ratio. The intermediate solution was diluted with 1X Assay Buffer in 1:80 ratio. 50  $\mu$ L cell suspension was mixed with 150  $\mu$ L working

solution and incubated at 37°C for 30 minutes. ROS activated cells were counted with Muse Cell Analyzer (Merck Millipore).

## 2.15 DNA Repair Genes Expression upon Inhibitor Treatment

### 2.15.1 Drug Treatment

$3 \times 10^5$  THP-1 cells were seeded in 6 well plates and treated with 1  $\mu$ M of indicated inhibitors (AD-94, MH-25, AD-58) for 72 hours.

### 2.15.2 RNA Extraction

RNA extraction was performed as described in Chapter 2.11.2.

### 2.15.3 cDNA Synthesis

cDNA Synthesis was performed as described in Chapter 2.11.3.

### 2.15.4 RT-qPCR

Gene expression levels were quantified with RT-qPCR analysis using the same protocols as described in Chapter 2.11.

**Table 2.3:** DNA repair genes and specific primer sequences.

| Gene    | Sequence (5'-3')            |
|---------|-----------------------------|
| OGG1    | F: GCTCAGAAATTCCAAGGTGTG    |
|         | R: GACATCATCAAGCTGGATGAG    |
| MUTYH   | F: CATATCAAGTATATGGGCTGGC   |
|         | R: CTTTTGGAACCCATACAGGTC    |
| GADD45A | F: GCCCAATTAGTGTCGTGCG      |
|         | R: TTCTGCACTCACTCACAGGC     |
| XPA     | F: CTTGTGATAACTGCAGAGATGC   |
|         | R: CAATAAATTTAAGAGGTGGCTCTC |
| ERCC1   | F: CCAGGTGGATGTGAAAGATCC    |

|       |                             |
|-------|-----------------------------|
|       | R: CTTGTAGGTCTCCAGGTACC     |
| ERCC3 | F: TGCTGTACACCATGAACCCC     |
|       | R: CCCTGAGACGTAGGTCCGTA     |
| RAD51 | F: TTGAAGCAAATGCAGATACTTCAG |
|       | R: GGCATTTATGCCACACTGCTC    |
| RAD52 | F: AGAAGGTGTGCTACATTGAGG    |
|       | R: GATATGAACCATCCTTCAGCTG   |
| ATM   | F: TTCCATCGCATGTGATTAAAGCA  |
|       | R: AATTTTCTGATAGGAATCAGGGC  |
| ATR   | F: ACCAATTGTGGAGGAGATTTC    |
|       | R: ACTTCTGAGAACTCTTGTATCTG  |
| XRCC5 | F: TATTTTCAGTGTCTGCTGCACAG  |
|       | R: GGGAGGATTCAGCATATTCCA    |
| LIG4  | F: GTAGTTTCTTGTTTAGCTGAGGG  |
|       | R: TGCAACACGACTATGATCTTCC   |
| GAPDH | F: CTGACTTCAACAGCGACACC     |
|       | R: GTTGTCATACCAGGAAATGAGC   |

## 2.16 RNA-seq

### 2.16.1 Drug Treatment

$3 \times 10^5$  THP-1 cells were seeded in 6 well plates and treated with 1  $\mu$ M of indicated inhibitors (AD-94, MH-25, AD-58, MH-18, and vehicle) for 72 hours.

### 2.16.2 RNA Extraction

RNA extraction was performed as described in Chapter 2.11.2.

### 2.16.3 RNA-Sequencing and Analysis

*\*RNA-Sequencing and bioinformatics analysis belongs to the hard work of Dr. Hamza Syed from the School of Medicine, Koç University.*

Poly-A capturing, library construction, and next-generation sequencing were performed by BGI Group (Hongkong, China). Sequencing was produced with 20 million readings/samples and 150 bp no stranded PE150 pair ended readings. RNA sequencing results were further analyzed by Dr. Hamza Syed with the software developed by him within the KUTTAM facility. Initially, the quality of the readings was evaluated with FastQC software for ensuring the quality control of the sequencing results (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Sequencing data were then matched using the most current reference genome (NCBI GRCh38) HiSAT2 (<http://daehwankimlab.github.io/hisat2/>) software. After the sequencing reading alignment, the reading counts of each gene were quantified using featureCounts (<http://bioinf.wehi.edu.au/featureCounts/>). The results were then analyzed using the R package DESeq2 (<https://bioconductor.org/packages/release/bioc/html/DESeq2.html>) for the identification of differentially expressed genes. Significant expression differences were revealed by (<https://m.ensembl.org/info/data/biomart/index.html>). Finally, using the Gene Set Enrichment Analysis (<http://software.broadinstitute.org/gsea>) the pathways or gene networks of the differentially expressed genes were determined.

## 2.17 Validation of RNA-seq Hit Genes by RT-qPCR

### 2.17.1 cDNA Synthesis

cDNA synthesis was performed as described in Chapter 2.11.3.

### 2.17.2 RT-qPCR

Gene expression levels were quantified/validated with RT-qPCR analysis using the same protocols as described in Chapter 2.11.

**Table 2.4:** RNA-seq hit Differentially expressed genes and specific primer sequences.

| Gene  | Sequence (5'-3')        |
|-------|-------------------------|
| CD163 | F: GGGCTAATTCCAGTGCAGGT |
|       | R: GCTGACTCATTCCCACGACA |
|       | F: ATGTGAGAGTCCTCCTCGGT |

|                   |                             |
|-------------------|-----------------------------|
| FRCLA             | R: TCAGCAGTAGCCTTTGTGGT     |
| PRTN3             | F: CGGCCACATAACATTTGCACT    |
|                   | R: GCACATCCCCAGATCACGAA     |
| S100A12           | F: CACTGCTGGCTTTTTGCTGT     |
|                   | R: TGTCAAAATGCCCCTTCCGA     |
| RGMB              | F: AAACGTACCTGTGGTCCCTG     |
|                   | R: ATACGCAGGCTCTTGGCATC     |
| LY96              | F: AGGGAGAGACTGTGAATACAACAA |
|                   | R: GGGCTCCCAGAAATAGCTTCA    |
| MPEG1             | F: ATGGGCTAAATCAGGCAAGC     |
|                   | R: ACTGTCCATCCTCTGTTGTCC    |
| GBP2              | F: CCAGGACGTTGGAGGGGTAA     |
|                   | R: GCCATGTCCAGGGTGTGTGT     |
| DTX4              | F: CCAGGGGTTTCCAACACAAG     |
|                   | R: TACAGATGGTGCAGTCCTCATC   |
| AGPAT9<br>(GPAT3) | F: AGGAACTACACTGGTTGGGC     |
|                   | R: TCTGGGGTCTGTACTGCTTGT    |
| CD53              | F: AGGGCAAGAATATCACGGCA     |
|                   | R: GACAAACACATTGCCAGCG      |
| KCTD12            | F: AGCCTCTTGTCCCACCTTG      |
|                   | R: TTCCTCTGGGTTCTCTCGGT     |
| LSP1              | F: ACAATACACCCAGGCCATCG     |
|                   | R: CCACCGACTCTTGGTACTGG     |
| HIPK2             | F: TCCTTTGTGGAGACTGAAGACAC  |
|                   | R: TCGCTCCCTTCCAAATCTGTC    |
| TBC1D4            | F: CAGTCTTTATGCTGCCCCCT     |
|                   | R: TCAGTAGGCTGAGTGCAACC     |
| GLIPR1            | F: AACTACGGACCAGGAGGGAA     |
|                   | R: ACGTTTGACTTGGTCTCGCT     |
| ANXA2             | F: TCGGACACATCTGGTGACTTC    |
|                   | R: CGTCATAGAGATCCCGAGCAT    |
| LRP1              | F: ATGGTGCAGACGAGAGCATC     |
|                   | R: CAGGTCGGGTACTCACACTC     |
| LPXN              | F: TCACTGCGGAGAGGTGTTT      |
|                   | R: TGGGTGAGAACATGGCTAAGAA   |
| VIT               | F: GGTCCACTGATGGGTGTTGT     |
|                   | R: AAGGAGATGGCCCGACCTAC     |
| DDIT4             | F: GGTTTCGCACACCCATTCAAG    |
|                   | R: CAGGGCGTTTGCTGATGAAC     |
| IFI44             | F: TGAGTGAGAAAGAAGGCGGC     |
|                   | R: CCTTCAGCGATGGGGAATCA     |

|          |                            |
|----------|----------------------------|
| FLJ36000 | F: AAGAGGAAGCCGTGAATGGG    |
|          | R: ATAACAGGAGGGGTGCGAAG    |
| FNBP1L   | F: ACTGAACACCCCTTTTGGTT    |
|          | R: TCCTCAGAAACCCTCTTCAGGA  |
| RASGRP2  | F: TCGAGAAGATGGTGGAGTCTGT  |
|          | R: CTGGAGCGCAGGAAATAGGA    |
| SNX7     | F: CCCTGAAAGCAGATTGGGAGA   |
|          | R: ATGACTCCCACGTAGCAAGG    |
| BTG1     | F: ATCTCCAAGTTTCTCCGCACC   |
|          | R: CTTGCATGGCTTTTCTGGGA    |
| IFI44L   | F: TTGGAAGTGGACCCCATGAAG   |
|          | R: AATGCAGGGCTGTAACGCT     |
| GAPDH    | F: CTGACTTCAACAGCGACACC    |
|          | R: GTTGTTCATACCAGGAAATGAGC |

### 2.18 Statistical Analysis

All normalizations were performed between vehicle and treatment groups using GraphPad Prism version 8.0 and Microsoft Excel 2018.

Significance analyses were performed with student's t-test, one or two-way ANOVA.

|     |                |
|-----|----------------|
| ns  | $p > 0.05$     |
| *   | $p \leq 0.05$  |
| **  | $p \leq 0.01$  |
| *** | $p \leq 0.001$ |

## Chapter 3:

## RESULTS

3.1 *Synthesis of Novel Inhibitors*3.1.1 *AD-94 is a Novel LSD1 Inhibitor*

AD-94/iLSD1 was developed for selectively targeting LSD1 based on the structure of GSK2879552, which is a known selective LSD1 inhibitor. To increase target engagement, the carboxylic acid of GSK2879552 was removed (**Figure 3.1**). The catalytically inactive isomer of GSK2879552 (MH-18/iNeg.Cntrl), where both substitutions are on the same carbon of the cyclopropyl ring, was used as a negative control (**Figure 3.1**). The activity of these compounds was initially tested using in vitro enzyme activity tests and the IC<sub>50</sub> values for LSD1 inhibition were calculated (**Table 3.1**).

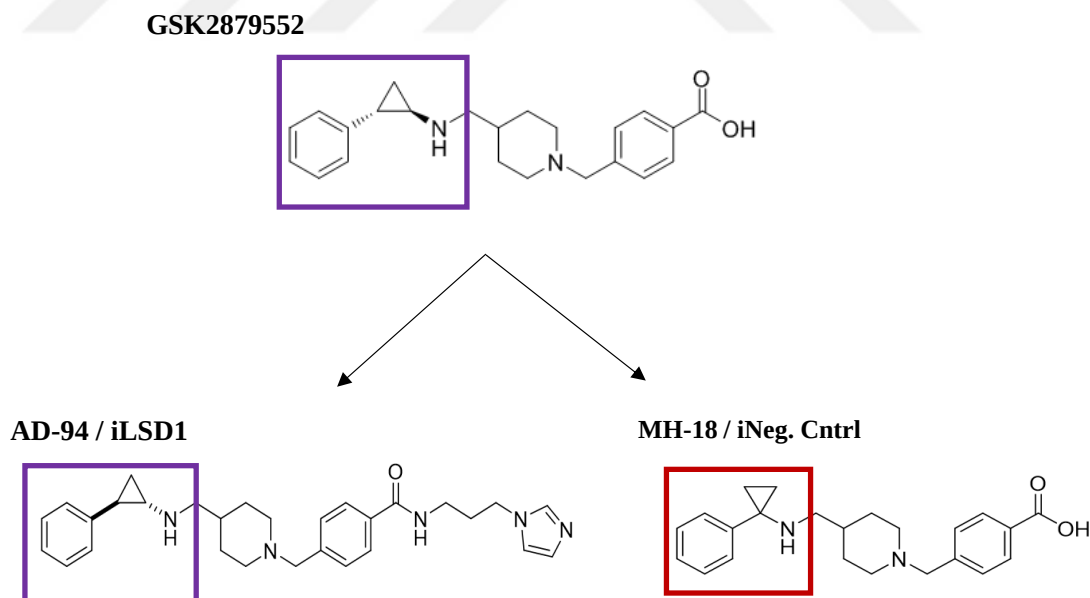


**Figure 3.1:** Chemical structure of novel LSD1 inhibitor AD-94 and negative control MH-18

Purple squares indicate the active sites for LSD1 binding, red square indicates the inactive site for LSD1 due to cyclopropyl amine ring configuration.

The figure is adapted from Lee et al. University of East Anglia, 2020 (unpublished data)

### 3.1.2 MH-25 is a Novel HDAC6 Inhibitor

To generate an HDAC6 inhibitor, the carboxylic acid in MH-18 (negative control compound) was converted into hydroxamic acid, which generated an active pocket for the  $\text{Zn}^{2+}$  cofactor in the zinc-dependent HDACs, similar to Ricolinostat, which is a known HDAC6 inhibitor (**Figure 3.2**). Indeed, in vitro enzyme activity tests confirmed that this novel compound (MH-25) effectively inhibited HDAC6 activity and was several folds more selective towards HDAC6 compared to other HDACs (namely HDAC1 and 3) (**Table 3.1**).

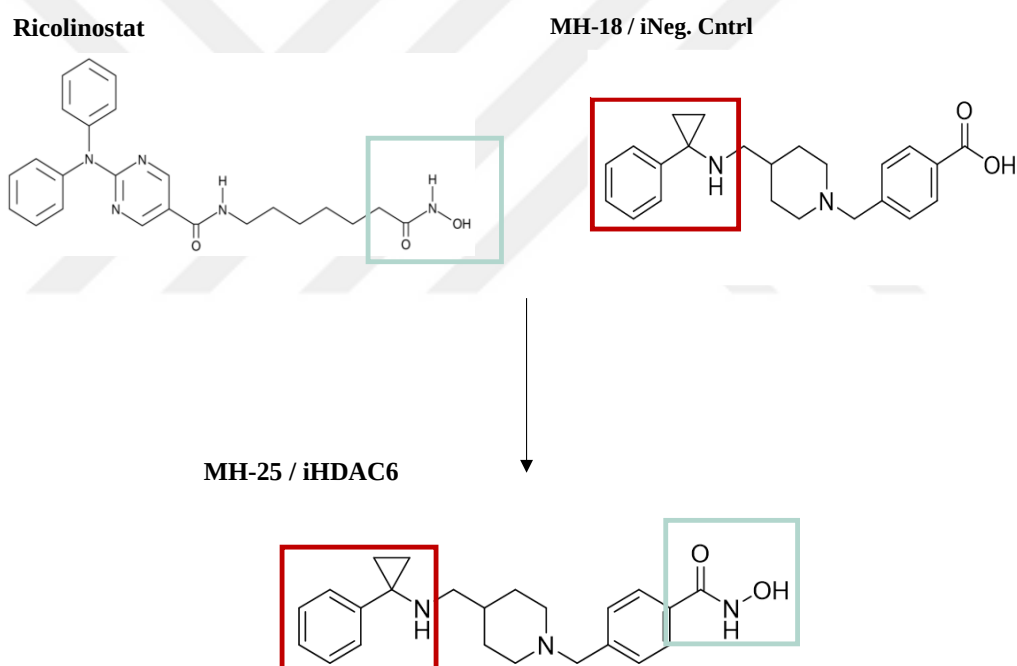


**Figure 3.2:** Chemical structure of novel HDAC inhibitor MH-25

Blue squares indicate the active sites for HDAC6 binding pocket, red square indicates inactive site for LSD1 binding due to cyclopropyl amine ring configuration

Figure adapted from Lee et al. University of East Anglia, 2020 (unpublished data).

### 3.1.3 AD-58 is a Novel LSD1/HDAC6 Dual Inhibitor

To generate a dual inhibitor that targets both LSD1 and HDAC6, the carboxylic acid of GSK2879552 was converted to hydroxamic acid to synthesize AD-58/iDual. GSK2879552 also contains a functional carboxylic group, which allows easy conversion to a hydroxamic acid and therefore, would be most likely to bind to the  $\text{Zn}^{+2}$  cofactor in the Zinc-dependent HDAC6 (**Figure 3.3**). Consistent with this prediction, AD-58 successfully inhibited both LSD1 and HDAC6 enzymes as tested by *in-vitro* enzyme activity assays.

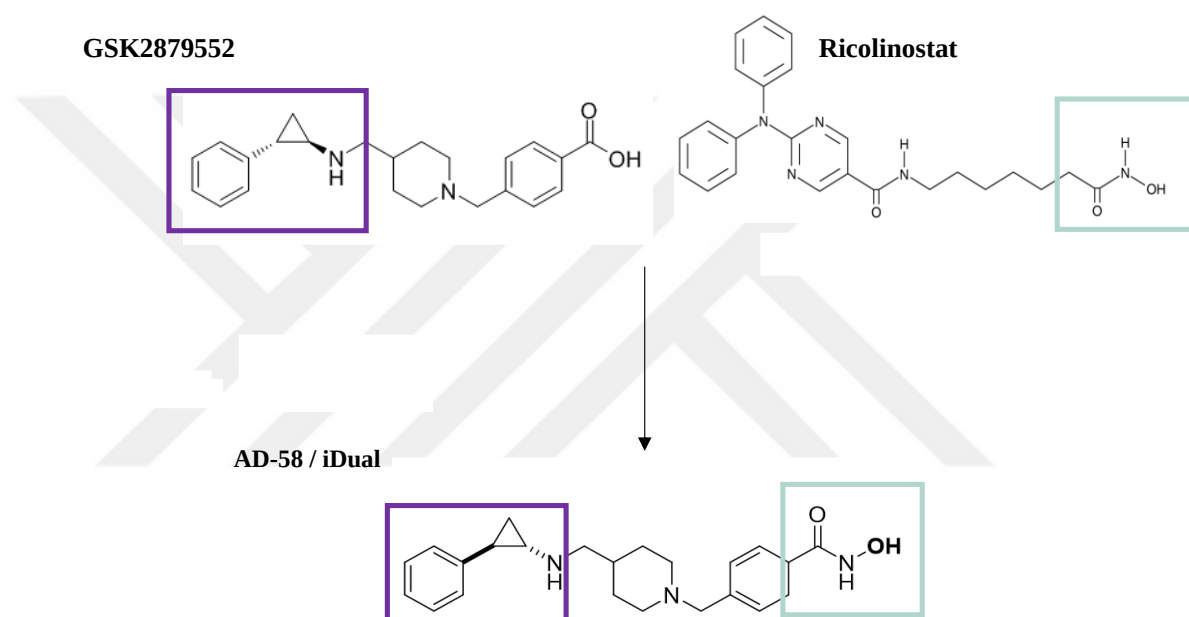


**Figure 3.3:** Chemical structure of novel dual inhibitor AD-58

Purple squares indicate the active sites for LSD1 binding, blue squares indicate the active sites for HDAC6 binding pocket.

Figure adapted from Lee et al. University of East Anglia, 2020 (unpublished data).

## 3.2 *In-vitro* Enzyme Activities of Novel Inhibitors

To determine whether the compounds exhibited the predicted inhibition of their target, the activity of LSD1 and HDACs inhibitors were measured by kinetic *in-vitro* enzyme activity assays. The serial dilutions of the different concentrations of the inhibitors were incubated with the indicated enzymes and their corresponding substrates,

and the enzyme activity was measured as a colorimetric readout and % inhibition and half inhibitory concentrations (IC<sub>50</sub>) were calculated for all wells.

**Table 3.1:** IC<sub>50</sub> values of novel inhibitors and positive control GSK2879552 for LSD1, HDAC6, HDAC1, and HDAC3 in kinetic enzyme assays.

| <u>Compounds</u>  | IC <sub>50</sub> ( $\mu$ M) |              |            |            |
|-------------------|-----------------------------|--------------|------------|------------|
|                   | LSD1                        | HDAC6        | HDAC1      | HDAC3      |
| GSK2879552/iLSD1  | <b>1.68</b>                 | Not active   | Not active | Not active |
| AD-94/iLSD1       | <b>0.06</b>                 | Not active   | Not active | Not active |
| MH-25/iHDAC6      | Not active                  | <b>0.248</b> | 7.57       | 4.09       |
| AD-58/iDual       | <b>0.551</b>                | <b>0.140</b> | 5.04       | 2.28       |
| MH-18/iNeg. Cntrl | Not active                  | Not active   | Not active | Not active |

We found that both AD-94/iLSD1 and AD-58/iDual was more specific for inhibiting LSD1 when compared to the reference compound (GSK2879552) as determined by the LSD1 enzyme activity assay (25-fold and 3 fold more specificity, respectively). As expected, MH-25/iHDAC6 and negative control MH-18 did not inhibit LSD1 activity, indicating that cyclopropyl ring disruption impairs the ability of binding to LSD1.

HDAC enzyme activity assay showed that both MH-25/iHDAC6 and AD-58/iDual had an affinity to inhibit HDAC6, while AD-58/iDual had 2-fold selectivity for inhibiting HDAC6 enzyme compared to MH-25/iHDAC6. This data suggested that the addition of hydroxamic acid increased the ability of binding HDAC active pocket. As expected, compounds which were not designed to inhibit HDAC6 (GSK2879552, negative control MH18, and AD-94/iLSD1) showed no inhibition, whilst MH-25/iHDAC6 and AD-58/iDual had a low level of affinity for HDAC1 and HDAC3 enzymes. On the other hand, MH-25/iHDAC6 showed 30-fold selectivity for HDAC6 compared to HDAC1, and 16-fold selectivity compared to HDAC3. Finally, AD-58/iDual had 35-fold selectivity for HDAC6 compared to HDAC1 and 16-fold selectivity compared to HDAC3.

This data suggested that the novel compounds successfully inhibited the targeted enzymes as assessed by the *in-vitro* enzyme activity assays. As expected, negative control MH-18 did not show any affinity for indicated enzymes.

### **3.3 *Inhibitors Stabilize Target Enzymes in THP-1 cells: CETSA***

The cellular thermal shift assay (CETSA) is used for evaluating drug-protein interactions in cells and allows us to monitor the thermal stabilization of a protein bound to a ligand. The procedure relies on the strength of the interaction between the target and the tested inhibitor. In CETSA assay, compound treated cells and vehicle cells are subjected to a heat shock varying a range of temperatures using a thermal cycler. As the temperature applied to each sample increases, the target protein unfolds and aggregates. The temperature at which this aggregation takes place is a consequence of the intrinsic thermal stability of the relevant protein within the cell. Following the heat shock process, cells are lysed and the thermostabilized proteins and aggregated fractions are physically separated by centrifugation. At the final step, the detection of the remaining thermostabilized target protein is performed by, gel electrophoresis and western blotting with target-specific antibodies. Upon target engagement of the drug, the target protein becomes more stable, and unfolding requires higher temperatures due to additional bonding between the protein and the small molecule of interest. Thus, the obtained varying aggregation temperatures with and without drugs conditions can be compared, and the thermodynamic shifts demonstrate target and inhibitor engagement [Jafari et al., 2014].

For investigating target engagement upon inhibitor treatment, THP-1 cells were heated at the indicated temperatures, and cell lysates after the thermal stress were run on SDS gel and blotted with LSD1, HDAC6, HDAC1, and HDAC8 antibodies.

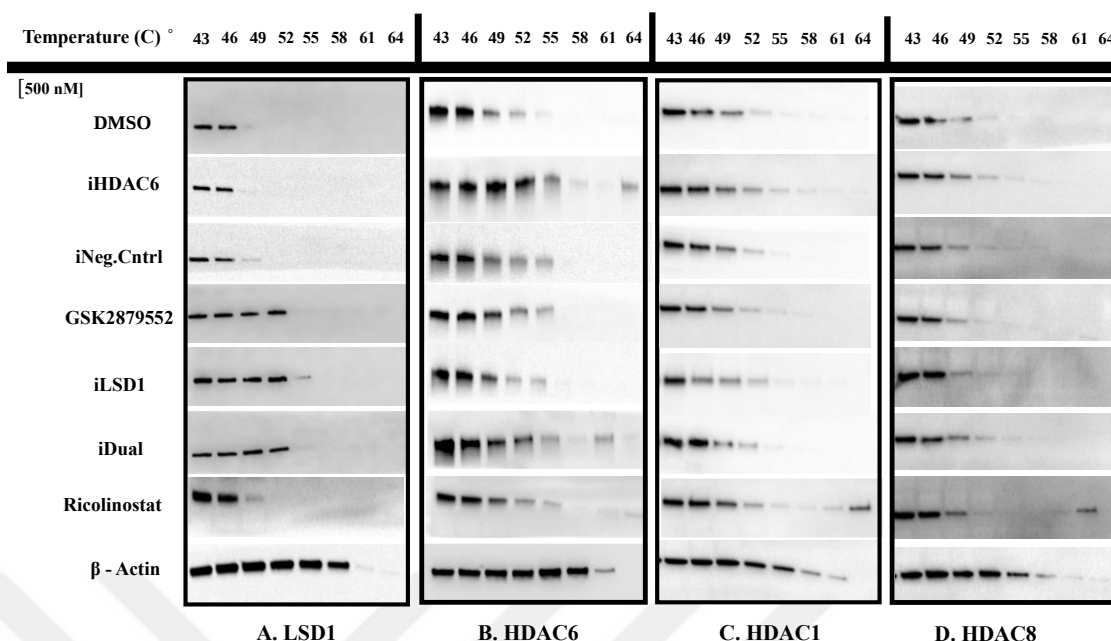


**Figure 3.4:** Target engagement upon inhibitor treatment in THP-1 cells

Stabilization of LSD1 (**A**), HDAC6 (**B**), HDAC1 (**C**), HDAC8 (**D**) was evaluated through degradation patterns in western blotting.

CETSA results indicated that the GSK2879552 and AD-58/iDual thermo-stabilized the enzyme up to 52 °C, while the thermostabilization preserved LSD1 structure up to 49 °C in the vehicle, negative control (MH-18), MH-25/iHDAC6 and Ricolinostat treated cells. Furthermore, novel inhibitor AD-94/iLSD1 addition led stabilization of LSD1 enzyme, up to 55 °C (**Figure 3.4A**).

HDAC6 is a relatively bigger protein that requires more energy to be unfolded, following that HDAC6 was aggregated after 55 °C in the vehicle, negative control (MH-18), GSK2879552, and iLSD1 treated cells. However, the majority of the HDAC6 enzymes were still thermostabilized at 64 °C in MH-25/iHDAC6, AD-58/iDual, and Ricolinostat treated cells (**Figure 3.4B**).

HDAC8 enzyme was found to be stabilized only in Ricolinostat treated cells, which suggests that MH-25/iHDAC6 and AD-58/iDual do not have target engagement against HDAC8 in THP-1 cells (**Figure 3.4D**). Similarly, Ricolinostat treatment stabilized the HDAC1 enzyme up to 64 °C (**Figure 3.4C**), while MH-25/iHDAC6 treatment showed a slight stabilization. As a result, AD-94/iLSD1 was found to stabilize LSD1, but not HDACs, as expected. MH-25/iHDAC6 induced thermal stabilization of HDAC6. AD-58/iDual showed target engagement against both LSD1 and HDAC6. Finally, negative

control MH-18 did not stabilize any of the indicated enzymes. Overall, these results strongly support that the novel inhibitors successfully interact and engage with their targets within the cell (**Figure 3.4**).

### 3.4 *LSD1 is Overexpressed in Leukemia*

LSD1 is highly expressed in many cancer types including hematopoietic malignancies [Karakaidos et al., 2019; Lynch et al., 2012]. Therefore, LSD1 expression levels were evaluated in different cancer cells by western blotting (**Figure 3.5A**). LSD1 expression has seen to be higher in several types of cancers compared to non-cancerous trophoblasts.

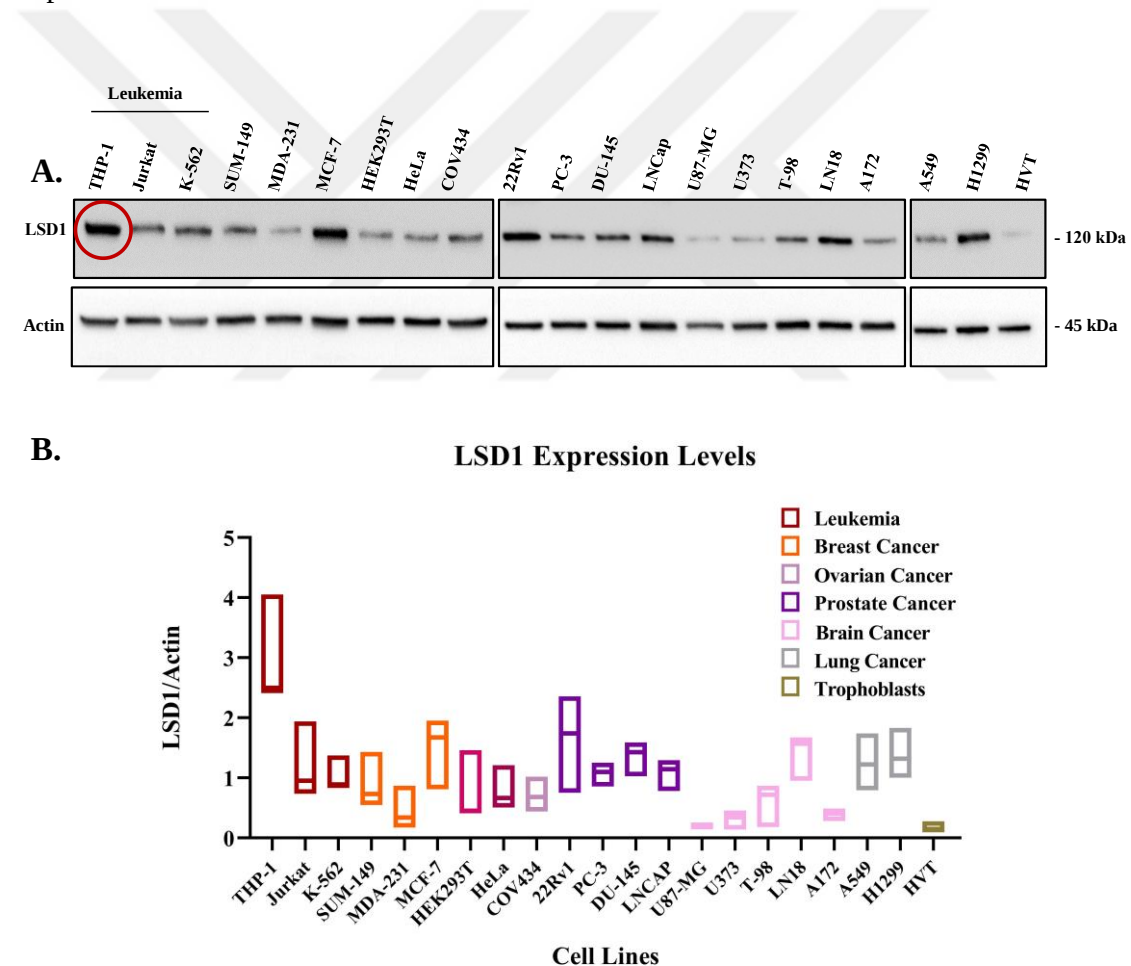


**Figure 3.5:** LSD1 expression levels in different cancer cell lines and non-cancerous HVT cell line

Western blot analysis (**A**) and quantification (**B**). Band intensities were quantified by Image J software.

The highest LSD1 expression was observed in leukemia cell lines followed by prostate and brain tumor cells (**Figure 3.5B**).

A further analysis was performed with two MLL-fused AML cells (THP-1/ MOLM-13), two non-fused CML (K-562), and T-cell leukemia (Jurkat) (**Figure 3.6**).

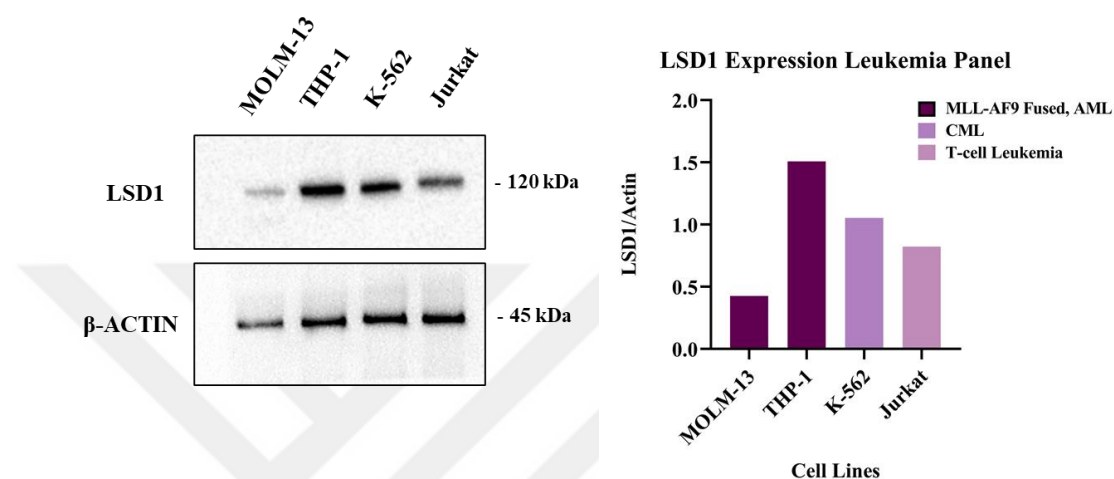


**Figure 3.6:** LSD1 expression levels in four different leukemia cells

Western blot analysis (**A**) and quantification (**B**). Band intensities were quantified by Image J software.

Although both THP-1 and MOLM-13 cell lines contain MLL-AF9 fusion, the THP-1 cell line showed a 3-fold higher LSD1 expression than MOLM-13 (**Figure 3.6B**). Therefore, THP-1 cells were selected for further studies of LSD1 inhibition.

### 3.5 *Tranylcypromine Significantly Reduces Growth of AML*

Tranylcypromine is a Monoamine oxidase (MAO) inhibitor, which also non-selectively inhibits LSD1 as well. Different cancer cells were evaluated based on their sensitivity to Tranylcypromine treatment. Indicated cell lines were treated with 2 mM of Tranylcypromine and cell viability was assessed with the SRB test (**Figure 3.7**).

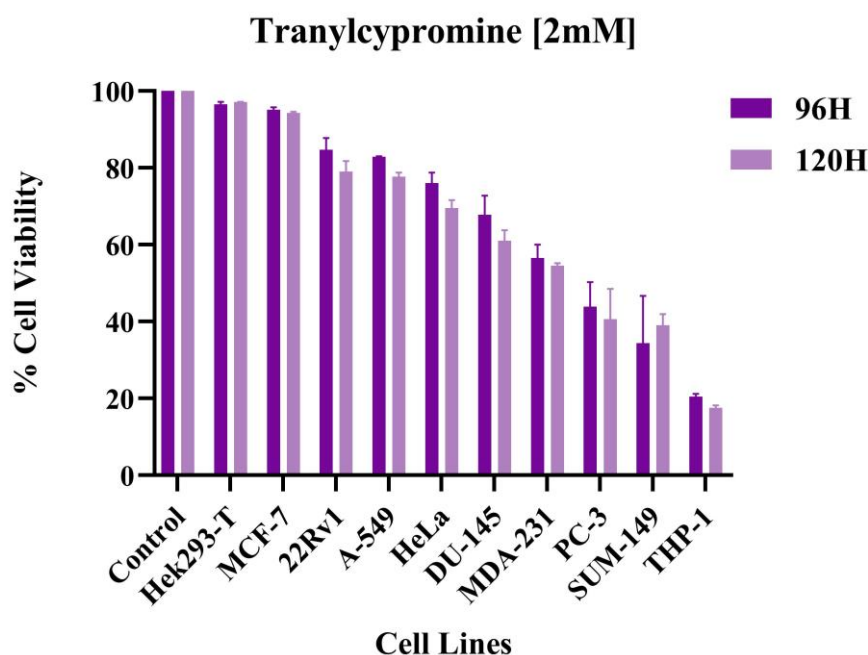


**Figure 3.7:** Cytotoxic effects of pharmacological inhibition of LSD1 via non-specific inhibitor Tranylcypromine on different cell lines

Cell viability was measured using the SRB test and normalized to untreated cells. Data represented as the average of  $\pm$  standard deviation from 3 biological repeats.

While HEK-293T and MCF-7 cells were not affected; LSD1 inhibition mildly (20%) reduced cell viability of 22Rv1, A549, and HeLa cells. Breast (MDA-231 and SUM-149) and prostate cancer (22Rv-1 and PC-3) cell growth was reduced upon Tranylcypromine treatment. However, the highest sensitivity upon LSD1 inhibition was observed on AML (THP-1) cells (**Figure 3.7**).

### **3.6 Pharmacological LSD1 and HDAC6 Inhibition Induced Cell Death in Leukemia Cells**

To examine possible toxic effects of LSD1 and HDAC6 inhibition on leukemic cells, THP-1, MOLM-13, K-562, and Jurkat cells were treated for 5 days with GSK2879552, AD-94/LSD1 inhibitor, AD-58/iDual, MH-25/iHDAC6, Ricolinostat, and negative control MH-18, following that cell viability was measured with ATP-dependent CTG Assay.

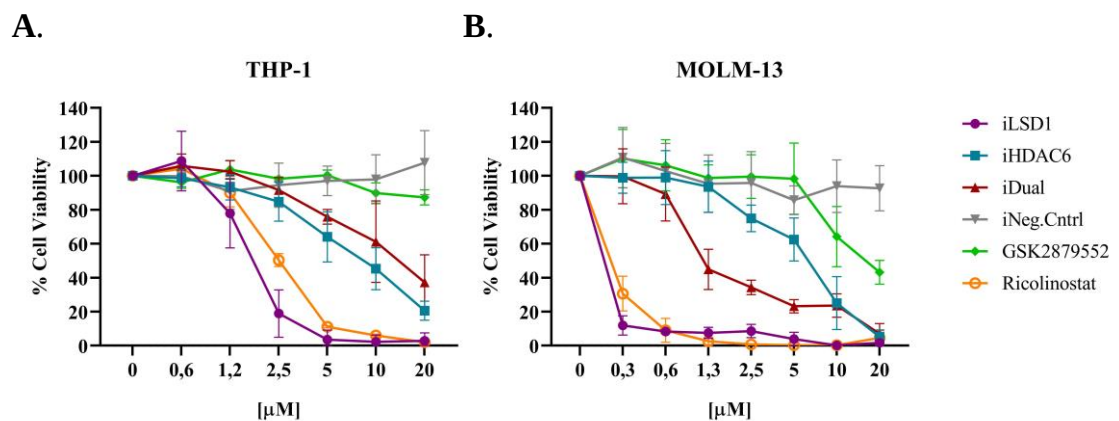


**Figure 3.8:** Cytotoxic effects of novel inhibitors in MLL-fused AML cell lines

Cell viability curves of THP-1 (**A**) and MOLM-13 (**B**) cells in response to the inhibitors. Cell viability was measured using ATP-dependent CTG assay and normalized to control cells. Data represented as the average of  $\pm$  standard deviation from 3 biological repeats.

Negative control (MH-18) showed no reduction of cell viability in four cell lines, supporting that it has no activity for indicated enzymes (**Figure 3.8** and **Figure 3.9**). LSD1 inhibition with GSK2879552 decreased cell viability of MOLM-13 cells to 40%, whereas no cell death was observed on THP-1 cells. On the other hand, LSD1 inhibition with AD-94/iLSD1 induced substantial cell death on both cell lines, even at very low doses. Similarly, HDAC6 inhibition with Ricolinostat severely decreased cell viability on both cell lines. MH-25/iHDAC6 and AD-58/iDual treatments showed gradual cell death on both cell lines, while MOLM-13 cells were more sensitive to both inhibitions (**Figure 3.8**).

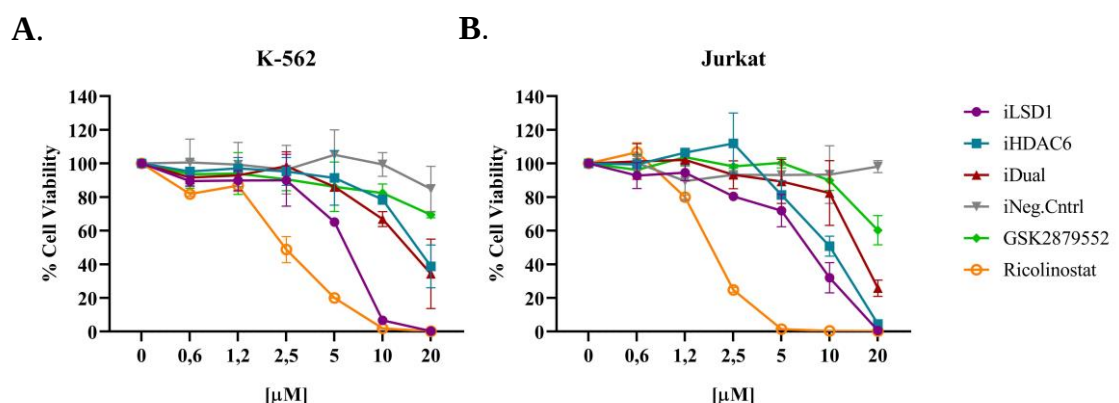


**Figure 3.9:** Cytotoxic effects of novel inhibitors in non-MLL-fused CML and T-cell leukemia cell lines

Cell viability curves of K-562 (**A**) and Jurkat (**B**) cells in response to the inhibitors. Cell viability was measured using ATP-dependent CTG assay and normalized to control cells. Data represented as the average of  $\pm$  standard deviation from 3 biological repeats.

Novel inhibitors induced cell death in non-MLL fused cell lines but with lower efficacies. LSD1 inhibition with GSK2879552 decreased cell viability of K-562 and Jurkat to 80% and 60%, respectively. HDAC6 inhibition with Ricolinostat showed similar cell viability results with MLL-fused cells. The loss of cell viability as a result of HDAC6 inhibition with Ricolinostat was similar to MLL-AF9 fusion cells. LSD1 inhibition with AD-94/iLSD1 indicated gradual cell death on both cell lines, yet K-562 cells were slightly more sensitive. HDAC6 inhibition with MH-25/iHDAC6 resulted in the complete death of Jurkat cells at 20  $\mu$ M, while cell viability was 60% in the K-562 cell line at the same dose. Both enzyme inhibition with AD-58/iDual, reduced cell viability on two cells gradually, whereas Jurkat cells were slightly more sensitive (**Figure 3.9**).

**Table 3.2:** Half inhibitory concentration ( $IC_{50}$ ) values of inhibitors on four different leukemia lines

| Compounds    | $IC_{50}(\mu M)$ |                  |                  |                  |
|--------------|------------------|------------------|------------------|------------------|
|              | THP-1            | MOLM-13          | K-562            | Jurkat           |
| GSK2879552   | >20              | 14.97 $\pm$ 3.48 | >20              | >20              |
| iLSD1        | 1.81 $\pm$ 0.32  | 0.003 $\pm$ 0.88 | 6.15 $\pm$ 0.27  | 7.73 $\pm$ 0.82  |
| iHDAC6       | 9.68 $\pm$ 2.39  | 6.66 $\pm$ 1.55  | 17.6 $\pm$ 1.61  | 10.72 $\pm$ 0.80 |
| iDual        | 16.34 $\pm$ 5.42 | 1.29 $\pm$ 0.36  | 16.64 $\pm$ 2.65 | 15.46 $\pm$ 1.42 |
| iNeg.Cntrl   | >20              | >20              | >20              | >20              |
| Ricolinostat | 2.91 $\pm$ 0.14  | 0.22 $\pm$ 0.03  | 2.55 $\pm$ 0.25  | 1.92 $\pm$ 0.003 |

As shown in (**Table 3.2**), the MOLM-13 cell line showed higher sensitivity to all inhibitors under examination. This sensitivity may be due to the biological differences of this cell line, rather than its dependence on LSD1 or HDAC6. Comparing  $IC_{50}$  values, AD-94/iLSD1 was more effective on THP-1 cells (3.5- 4-fold against K-562 and Jurkat cells). On the other hand, MH-25/iHDAC6 showed lower  $IC_{50}$  values on both THP-1 and Jurkat cells, whereas K-562 cells were observed to be insensitive. Finally, AD-58/iDual similarly inhibited cell growth on THP-1, K-562, and Jurkat cells.

### 3.7 Pharmacological Inhibition of LSD1 Induces Global H3K Methylation

LSD1 removes one or two methyl groups from H3 lysine 4 and lysine 9 via a redox reaction [Chen et al., 2012]. Therefore, inhibition of enzymatic LSD1 activity leads to higher methylation levels on LSD1 targets.

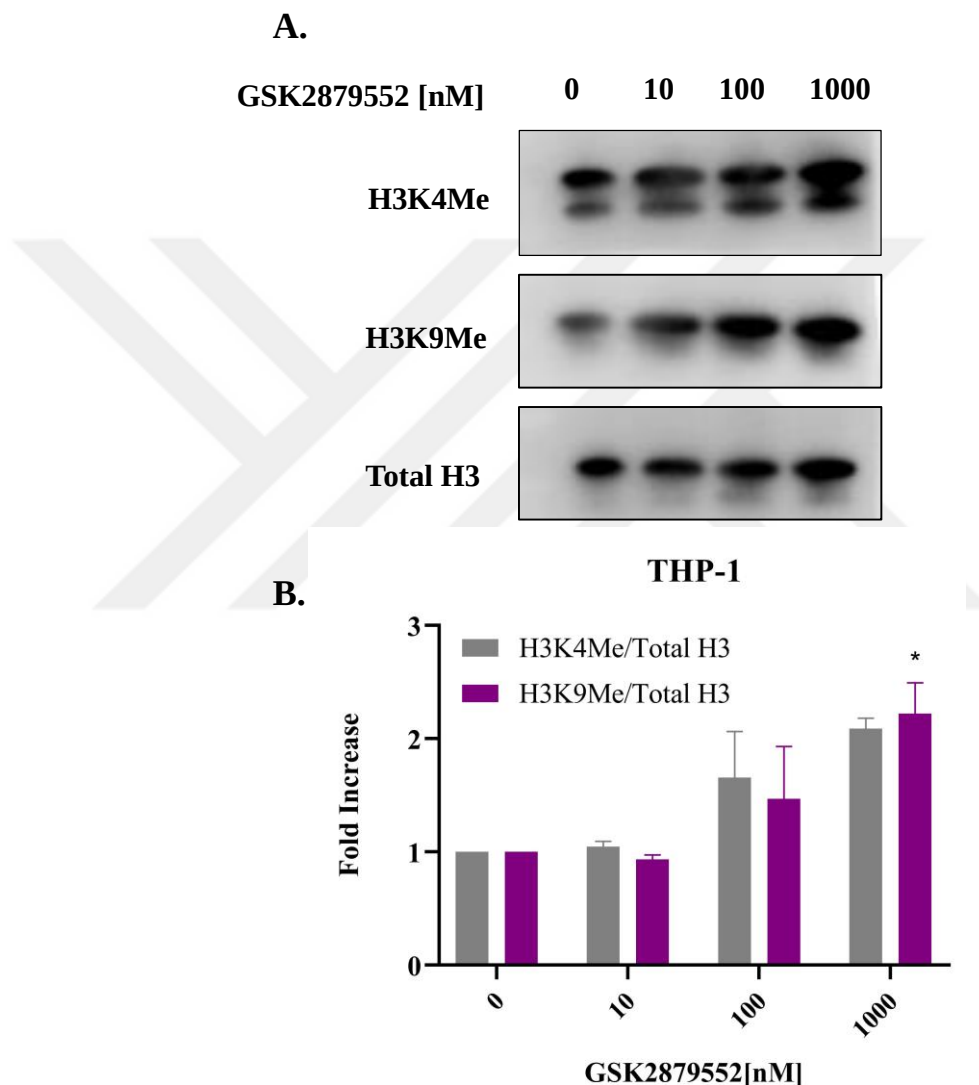


**Figure 3.10:** Increased histone methylation levels upon LSD1 inhibition with Tranylcypromine

Western blot analysis (**A**) of H3K4 and H3K9 methylation upon Tranylcypromine treatment for 24 h and quantification (**B**). Band intensities were quantified by Image J software. H3K4 and H3K9 methylation expressions were normalized to Total H3 expressions. Data represented as the average of  $\pm$  standard deviation of 3 biological repeats (\* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ , 2-way ANOVA).

To evaluate epigenetic changes on the histones in response to drug treatment, THP-1 cells were treated with varying concentrations (10, 100, 1000 nM) of GSK2879552. As expected, LSD1 inhibition with GSK2879552 increased H3K4 and H3K9 methylation in a dose-dependent manner (**Figure 3.10B**).

For the assessment of dimethylated H3K4 methylation, THP-1, and MOLM-13 cell lines were treated with 2  $\mu$ M of GSK2879552, AD-94/iLSD1 and AD-58/iDual for 24 hours (**Figure 3.11**).

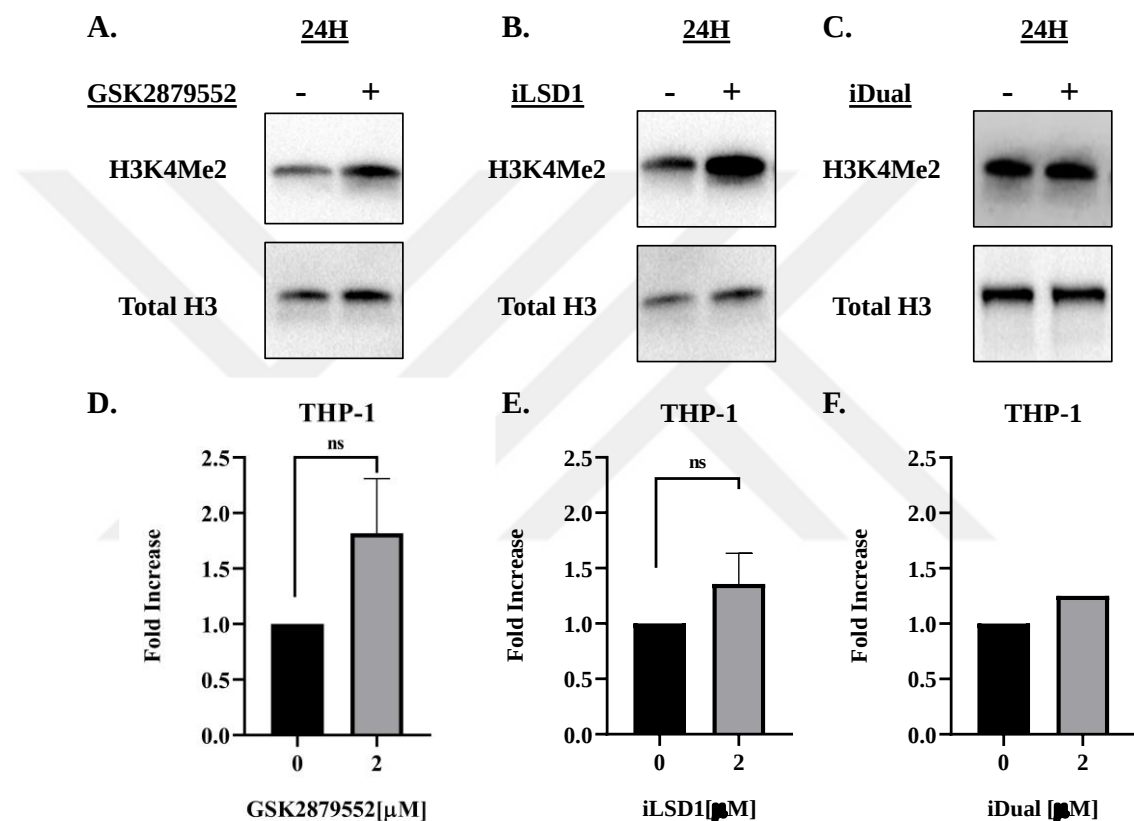


**Figure 3.11:** H3K4 dimethylation levels upon LSD1 inhibition with GSK2879552, AD-94, and AD-58 in THP-1 cells

Western blot analysis of H3K4 dimethylation upon GSK2879552 (**A**), AD-94/iLSD1 (**B**), AD-58/iDual (**C**) for 24 h in THP-1 cell lines. Western blot quantification (**D**, **E**, and **F**). Band intensities were quantified by Image J software. H3K4 dimethylation expressions were normalized to Total H3 expressions. Data represented as average of  $\pm$  standard deviation of 2 biological repeats (\* $p$ <0.05, \*\* $p$ <0.01 and \*\*\* $p$ <0.001, t-test).

Global H3K4me2 expression was only slightly increased upon 2  $\mu$ M of indicated inhibitors compared to the control group in THP-1 cells (**Figure 3.11**).

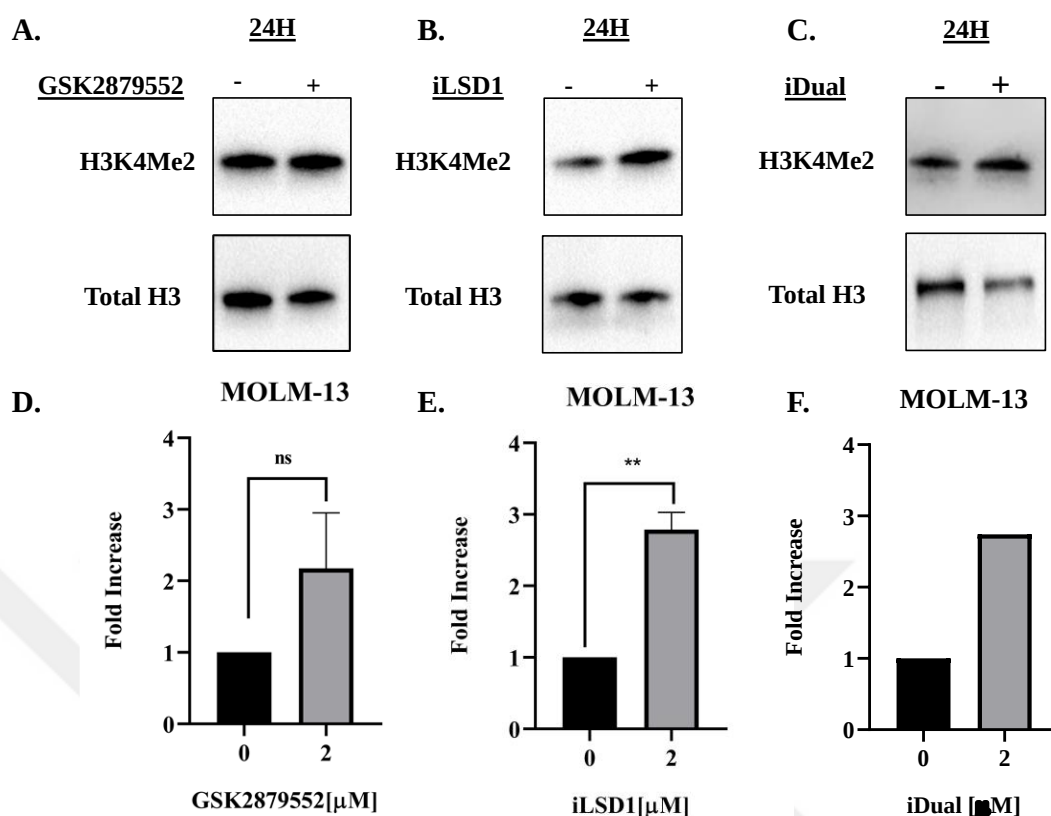


**Figure 3.12:** H3K4 dimethylation levels upon LSD1 inhibition with GSK2879552, AD-94, and AD-58 in MOLM-13 cells

Western blot analysis of H3K4 dimethylation upon GSK2879552 (A), AD-94/iLSD1 (B), AD-58/iDual (C) for 24 h in MOLM-13 cell lines. Western blot quantification (D, E, and F). Band intensities were quantified by Image J software. H3K4 dimethylation expressions were normalized to Total H3 expressions. Data represented as average of  $\pm$  standard deviation of 2 biological repeats (\* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ , t-test).

On the other hand, global H3K4me2 expression was enhanced after treatment with GSK2879552 (Figure 3.12C). Treatment of MOLM-13 cells with AD-94/iLSD1 and AD-58/iDual significantly increased total H3K4me2 expression up to 3 and 2.7-fold in MOLM-13 cells (Figure 3.12D).

### 3.8 Pharmacological Inhibition of HDAC6 Induces Acetylated $\alpha$ -Tubulin

As mentioned earlier,  $\alpha$ -tubulin is an important HDAC6 target and reversible acetylation of  $\alpha$ -tubulin is associated with microtubule stability [Piperno et al., 1987]. THP-1 cells were treated with 4  $\mu$ M of MH-25/iHDAC6, AD-58/iDual, and Ricolinostat for 24 hours, and western blotting was performed to investigate acetylated  $\alpha$ -tubulin levels upon HDAC6 inhibition (Figure 3.13).

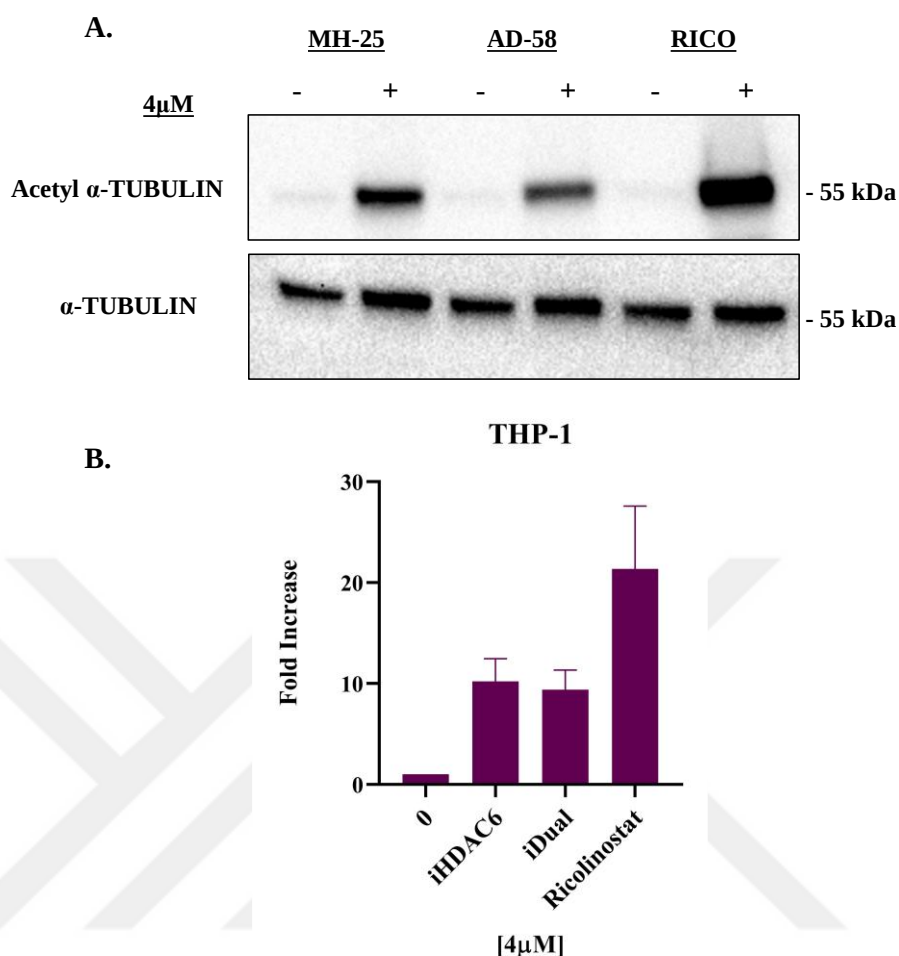


**Figure 3.13:** Acetylated  $\alpha$ -tubulin levels upon pharmacological inhibition of HDAC6

Western blot analysis (**A**) of acetylation of  $\alpha$ -tubulins and quantification (**B**) in THP-1 cell lines. Cells were treated with MH-25/iHDAC6, AD-58/iDual, and Ricolinostat for 24 h. Band intensities were quantified by Image J software. Acetylated  $\alpha$ -tubulin expressions were normalized to  $\alpha$ -tubulin expressions. Data represented as the average of  $\pm$  standard deviation of 2 biological repeats.

MH-25/iHDAC6 and AD-58/iDual treatments increased acetylated  $\alpha$ -tubulin up to 10-fold compared to non-treated cells. Accordingly, Ricolinostat treatment enhanced acetylated  $\alpha$ -tubulin levels up to 20-fold compared to control cells (**Figure 3.13B**). This data suggested that HDAC6 inhibition upon inhibitor treatments leads to acetylation and stabilization of  $\alpha$ -tubulins.

### 3.9 LSD1 Inhibition Induces Macrophage Markers Expression

THP-1 is a human monocytic cell line derived from an AML patient. In a healthy individual, monocytes circulate through the blood and when they migrate into tissues, they differentiate into macrophages. In AML, monocyte differentiation is limited, thus cells grow constantly. LSD1 is known as one of the responsible enzymes for preventing macrophage commitment and differentiation [Fang et al., 2017]. Therefore, macrophage marker expressions were evaluated upon pharmacological LSD1 inhibition.

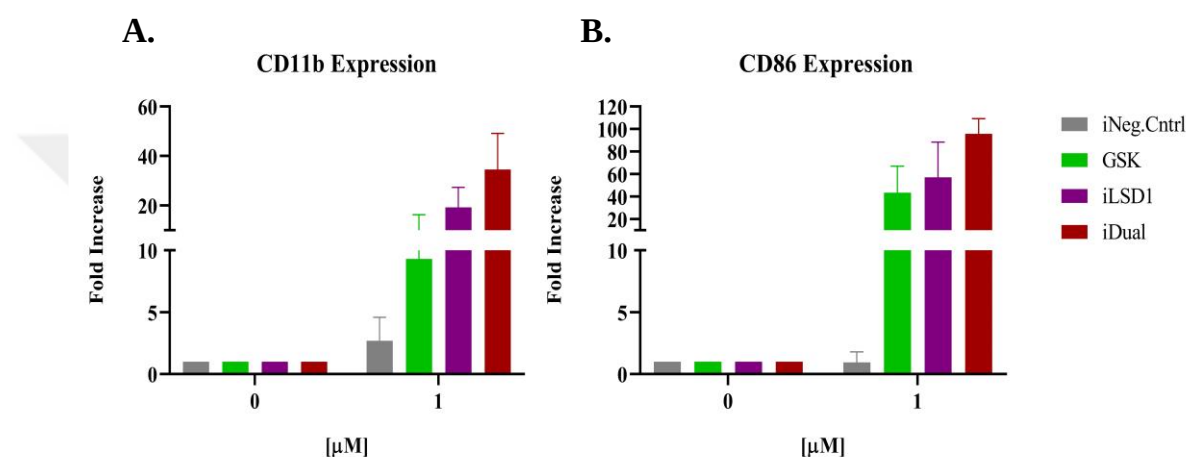


**Figure 3.14:** Macrophage marker expression upon LSD1 inhibition

RT-qPCR analysis of CD11b (A) and CD86 (B) mRNA levels upon LSD1 inhibition with GSK2879552, AD-94/iLSD1, AD-58/iDual for 24 h in THP-1 cells. Macrophage expression was normalized to two housekeeping genes,  $\beta$ -actin and GAPDH. Relative expression was determined with the  $2^{(-\Delta\Delta CT)}$  method. Data represented as the average of  $\pm$  standard error of the mean of 2 biological repeats with duplicates.

Two macrophage markers (CD11b and CD86) expression levels were investigated with RT-qPCR upon LSD1 inhibition in THP-1 cells (**Figure 3.14**). While there was no significant increase in the negative control (MH-18), CD11b and CD86 expressions were 10 and 40-fold higher in 1  $\mu$ M GSK2879552 treated cells. Similarly, 72 hours of treatment with AD-94/iLSD1 increased CD11b and CD86 expressions 20 and 60-fold, respectively. Finally, LSD1 inhibition with AD-58/iDual also induced expressions of two macrophage markers 30 and 100-fold when compared with the negative control. These results suggest that LSD1 inhibition promotes differentiation in THP-1 cells. While each molecule induces the expression of macrophage markers, the highest increase was observed in AD-58/iDual treatment.

### 3.10 The Cyclin-dependent Kinase Inhibitor p21 Expressions Upon Pharmacological HDAC6 Inhibition

p21 (CDKN1A) expression has been shown to increase after HDAC inhibition [Sun et al., 2019; Li et al., 2013]. p21 is a target for the p53 gene and therefore, associated with linking DNA damage and cell cycle arrest [Waldman et al., 1995; Bunz et al., 1998] and its expression has been known to be upregulated through p53-dependent cell cycle arrest following DNA damage [Bunz et al., 1998].

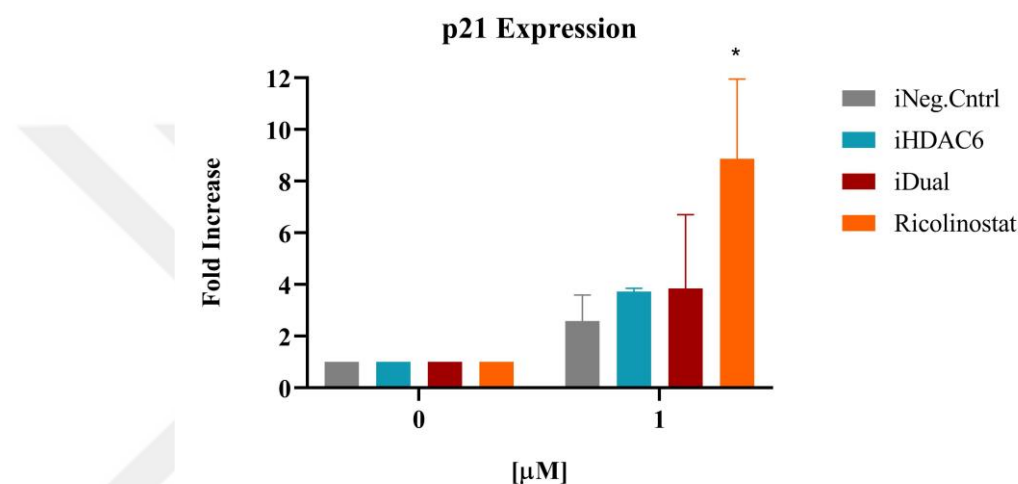


**Figure 3.15:** p21 expression levels in THP-1 cells after HDAC6 inhibition

RT-qPCR analysis of p21 mRNA levels upon LSD1 inhibition with MH-25/iHDAC6, AD-58/iDual, and Ricolinostat for 24 h in THP-1 cells. p21 expression was normalized to two housekeeping genes,  $\beta$ -actin, and GAPDH. Relative expression was determined with the  $2^{(-\Delta\Delta CT)}$  method. Data represented as the average of  $\pm$  standard error of the mean of 2 biological repeats with duplicates (\* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ , two-way ANOVA).

In order to evaluate p21 expressions in response to inhibitor treatment, THP-1 cells were treated with 1  $\mu$ M of the negative control (MH-18), MH-25/iHDAC6, AD-58/iDual, and Ricolinostat for 72 hours (**Figure 3.15**). As expected, RT-qPCR results confirmed that p21 expression was increased 8-fold in Ricolinostat treated cells. Similarly, p21 expression was elevated in cells following both MH-25/iHDAC6 and AD-58/iDual treatment albeit to a lesser degree (4-fold) (**Figure 3.15B**). Thus, the anti-cancer effect of inhibitors was further supported by growth inhibition through p21 expression.

### 3.11 Combination of Novel Inhibitors with Clinically Used Agents

*For all combination assays,*

*Sublethal doses of each drug were selected for drug combination assays. In this way, while the drugs were mildly effective in single treatments, the difference in cell viability as a result in combination become more apparent.*

Epigenetic drugs are usually not cytotoxic, and their promise is to prime the cells for subsequent therapies, usually by reprogramming cancer cells toward a normal state. In the course of our work, we exploited if any of the drugs under study can enhance the potency of clinically used drugs leading to synergy. Cytarabine is one of the primary drugs in AML, and the high dose of Cytarabine treatment is a highly effective method, which used frequently [Li et al., 2014]. Unfortunately, Cytarabine leads to serious side effects, therefore increasing the efficacy of the drug is an attractive study. For this reason, the effects of the combined treatment of novel inhibitors and Cytarabine have been studied. THP-1 cells were pre-treated with epigenetic inhibitors (1.25  $\mu$ M AD-94/iLSD1, 5  $\mu$ M MH-25/iHDAC6 and 5  $\mu$ M AD-58/iDual) for 72 hours and co-treated with Cytarabine (0.62  $\mu$ M) for 48 hours. Cytarabine, AD-94/iLSD1, MH-25/iHDAC6, AD-58/iDual single treatments did not decrease cell viability, yet no significant cell death was observed upon combination therapy (**Figure 3.16A**). Consequently, combined Cytarabine and LSD1 or HDAC6 inhibition did not reduce AML growth.

Cisplatin in conjunction with several drugs including Cytarabine, Etoposide, was reported to reduce AML. However, a combination of 3 cytotoxic drugs is not preferred due to possible side effects. Therefore, we examined the effects of Cisplatin combined with epigenetic inhibitors on AML. THP-1 cells were pre-treated with epigenetic inhibitors (1.25  $\mu$ M AD-94/iLSD1, 5  $\mu$ M MH-25/iHDAC6 and 5  $\mu$ M AD-58/iDual) for 72 hours and co-treated with Cisplatin (1.25  $\mu$ M) for 48 hours. Similar to our results with Cytarabine, treatment of THP-1 cells with a single drug, Cisplatin, AD-94/iLSD1, MH-25/iHDAC6, and AD-58/iDual, did not increase cell death more than 30%. There was no significant increase in cell death on combinatorial therapy treatment (**Figure 3.17**). Therefore, Cisplatin does not appear to synergize with any of the inhibitors under study.

To assess LSD1/HDAC6 inhibition sensitize AML cells against Paclitaxel we performed a combination assay with our inhibitors. Leukemic cells easily develop resistance to Taxane groups such as Paclitaxel, due to the overexpression of MDR1 gene

[Lopez et al., 2001]. An epigenetic regulation to reverse this state will undoubtedly increase the efficacy of Paclitaxel on AML cells. For this reason, THP-1 cells were treated with Paclitaxel and epigenetic inhibitors, and the effect of our epigenetic inhibitors was assessed for Paclitaxel sensitization or synergy. Cells were pre-treated with epigenetic inhibitors (1.25  $\mu$ M AD-94/iLSD1, 5  $\mu$ M MH-25/iHDAC6 and 5  $\mu$ M iDual) for 72 hours and co-treated with Paclitaxel (3  $\mu$ M) for 48 hours.

While Paclitaxel and AD-94/iLSD1 single treatments did not induce severe cell death, combination therapy significantly reduced cell viability to 45% ( $p \leq 0.01$ ) (**Figure 3.16C**).

On the other hand, MH-25/iHDAC6 and AD-58/iDual treatments alone did not decrease cell viability, and no significant increase in cell death was observed upon combination therapy with Paclitaxel (**Figure 3.16C**). Overall, AD94/iLSD1 and Paclitaxel combination treatment was promising, however MH-25/iHDAC6 and AD-58/iDual with Paclitaxel did not result in a positive synergy in AML cells.

Cytarabine treatment for 7 days and Doxorubicin treatment for 3 days are cornerstones of leukemia chemotherapy. Therefore, increasing the efficacy of Doxorubicin motivates researchers for clinical prospects in AML treatment. For this reason, we also examined Doxorubicin and LSD1 or HDAC6 inhibition in THP-1 cells. Cells were pre-treated with epigenetic inhibitors (1.25  $\mu$ M AD-94/iLSD1, 5  $\mu$ M MH-25/iHDAC6, and 5  $\mu$ M AD-58/iDual) for 72 hours and co-treated with Doxorubicin (62.5 nM) for 48 hours.

The first combination was performed with Doxorubicin and AD-94/iLSD1, as single treatments did not induce severe cell death, combination therapy significantly reduced cell viability to 30% ( $p \leq 0.01$ ) (**Figure 3.16D**).

Treatment of cells with MH-25/iHDAC6 and Doxorubicin treatments alone reduced cell viability by 30% and 25%, respectively. However, their combination led to a dramatic decrease in cell viability with only 30% of surviving cells (**Figure 3.16D**). Finally, AD-58/iDual and Doxorubicin treatment significantly reduced cell viability to 20% ( $p \leq 0.01$ ), while treatment with a single drug was mildly effective (**Figure 3.16D**). Altogether, these results indicated that pharmacological inhibition of LSD1 and HDAC6 sensitizes THP-1 cells to Doxorubicin treatment.

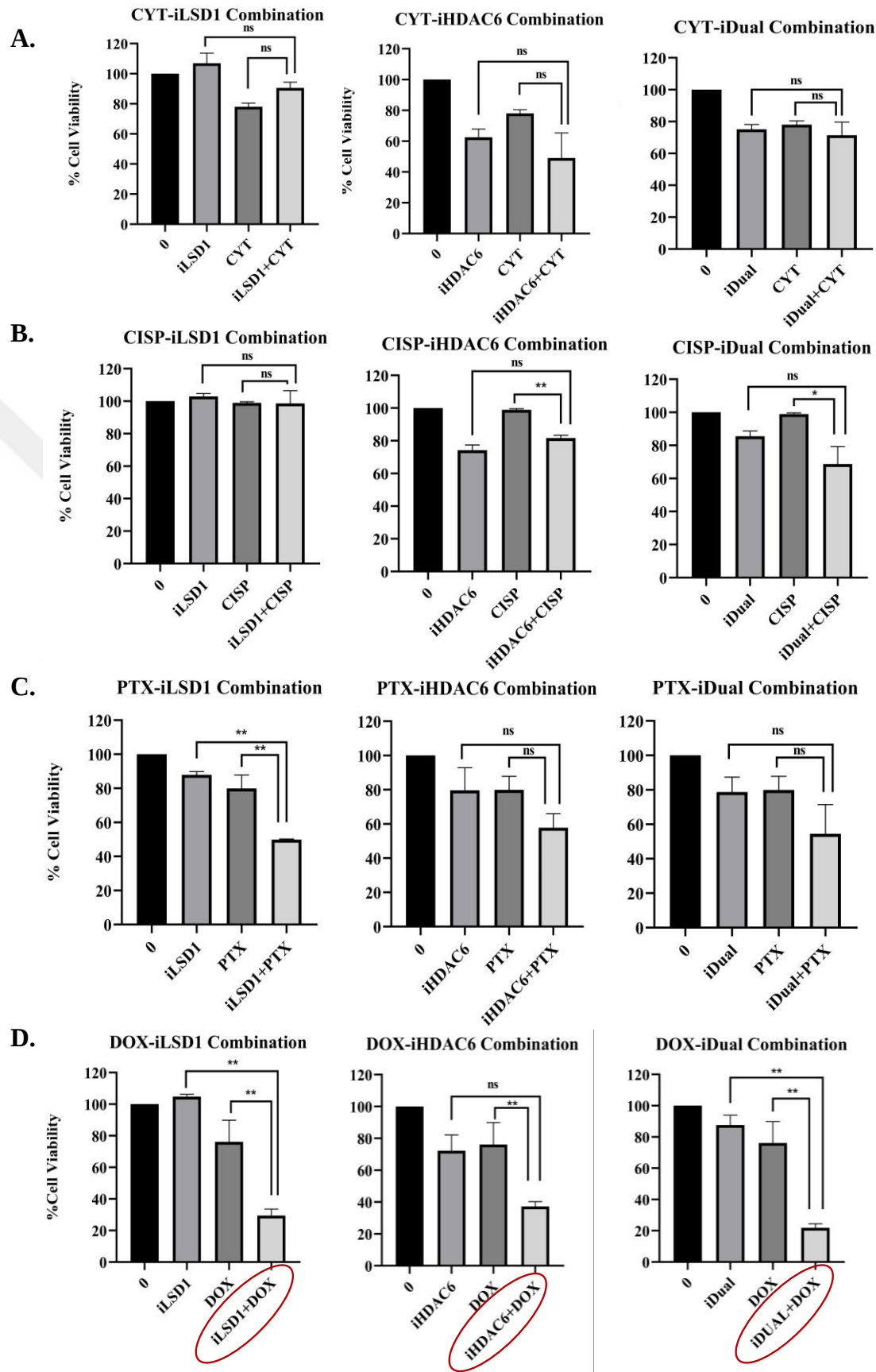


**Figure 3.16:** Cell viability in THP-1 cells after combination therapy with novel inhibitors and clinically used agents

THP-1 cells were pre-treated with AD-94/iLSD1, MH-25/iHDAC6, and AD-58/iDual for 72 h, then co-treated with Cytarabine (**A**), Cisplatin (**B**), Paclitaxel (**C**) Doxorubicin (**D**). Cell viability was measured using ATP-dependent Cell-Titer Glo assay and normalized to control cells. Data represented as the average of  $\pm$  standard deviation from 3 biological repeats (\* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ , Ordinary one-way ANOVA).

### ***3.12 Pharmacological LSD1 and HDAC6 inhibition with Doxorubicin Induces Apoptosis in AML cells***

#### ***3.12.1 Annexin V Staining***

Based on our previous findings, LSD1 and HDAC6 inhibition yielded enthralling results with Doxorubicin. These results prompted us to investigate the mechanism of cell death upon combination.

Phosphatidylserines (PS) are localized on the cell membrane facing the cell cytoplasm. In early apoptotic cells, membrane asymmetry is disrupted, thus the phosphatidylserines are translocated to the external side of the cell membrane [Lakshmanan and Batra, 2013]. Annexin V is an intracellular protein, which has an affinity to bind phosphatidylserines in a calcium-dependent manner. Fluorochrome-labeled Annexin V dye indicates early apoptotic cells by interacting with PSs. 7-AAD is a fluorescent DNA intercalator, which cannot pass through an intact cell membrane. Thus, it penetrates dead or damaged cells and excluded from live cells [Toba et al., 1996]. Therefore, co-staining Annexin V and 7-AAD ensure distinguishing live, apoptotic, and dead cells. Annexin V and 7-AAD positive THP-1 cells after combination treatment were determined by flow cytometry (**Figure 3.17**).

For this, THP-1 cells were pre-treated with epigenetic inhibitors (1.25  $\mu$ M of GSK2879552-iLSD1 and 5  $\mu$ M of GSK2879552-iHDAC6-iDual) for 72 hours and co-treated with Doxorubicin (62.5 nM) for 48 hours.

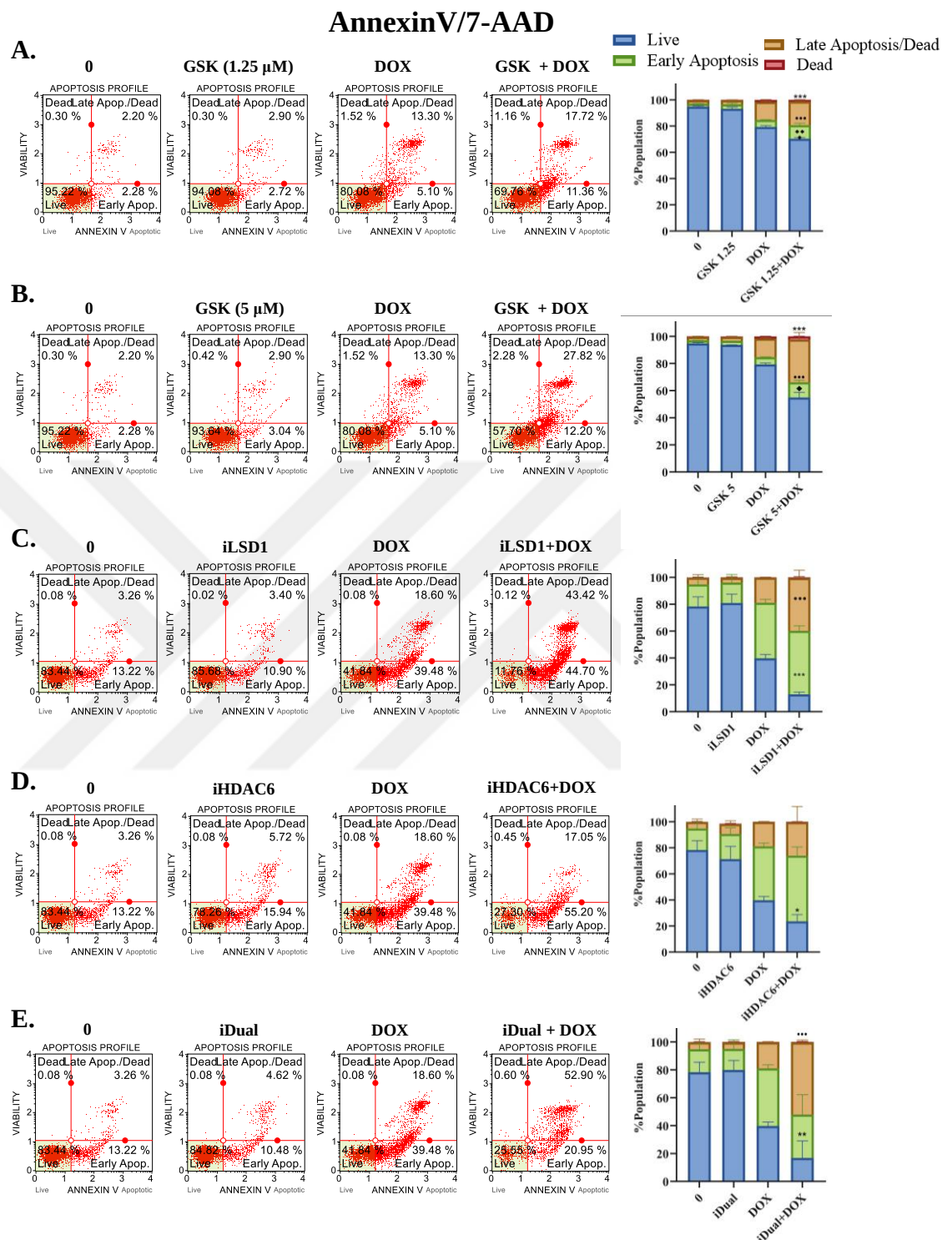


**Figure 3.17:** Analysis of Annexin V positive cells by flow cytometry after combination therapy

THP-1 cells were pre-treated with 1.25-5  $\mu$ M of GSK2879552 (**A** and **B**), 1.25  $\mu$ M of AD-94/iLSD1 (**C**), 5  $\mu$ M of MH-25/iHDAC6 (**D**) and 5  $\mu$ M of AD-58/iDual (**E**) for 72 h, then co-treated with 62.5 nM of Doxorubicin for 48h. Annexin V and 7-AAD positive cells were analyzed by flow cytometry. Data represented as the average of  $\pm$

standard deviation from 2 biological repeats (\* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ , two-way ANOVA).

Annexin V(-)/7-AAD(-): Live cells; Annexin V(+)/7-AAD(-): Early apoptotic cells; Annexin V(+)/7-AAD(+): Late apoptotic/dead cells; Annexin V(-)/7-AAD(+): Dead cells.

Two different doses of GSK2879552 were used in order to match the concentration of iLSD1, iHDAC6, or iDual. Flow cytometry analysis indicated that 1.25  $\mu\text{M}$  of GSK2879552 treatment alone, induced 5% of total apoptotic cells (early and late), and the Doxorubicin induced 18% Annexin V positive cells, whereas their combination resulted in 30% of total apoptotic cells (**Figure 3.17A**). GSK2879552 dose increase (5  $\mu\text{M}$ ), did not affect the rate of apoptosis alone, while Annexin V positive cells increased up to 40% in combination with Doxorubicin (**Figure 3.17B**). These results showed that GSK2879552 treatment sensitized AML cells against Doxorubicin in a dose-dependent manner.

Similar to GSK2879552, LSD1 inhibition with AD-94/iLSD1 and Doxorubicin induced 3% and 18% of late apoptotic cells, respectively. Their combination induced an explicit increase of total apoptotic cells 88% ( $p \leq 0.001$ ). In support of this, the proportion of living cells was only 10% ( $p \leq 0.001$ ) (**Figure 3.17C**).

HDAC6 inhibition with MH-25/iHDAC6 and combination with Doxorubicin treatment also induced Annexin V positive cells. While early apoptotic cells were 15% and 40% in iHDAC6 and Doxorubicin single treatments respectively, their combination resulted in 72% of total apoptotic cells (**Figure 3.17D**). Considering live and apoptotic cell rates upon Annexin V staining, HDAC6 and Doxorubicin treatment was not synergistic however, the co-treatment still had an anti-cancer effect on AML cells.

Simultaneous inhibition of LSD1/HDAC6 led 4% of late apoptotic cells and a significant increase was observed upon Doxorubicin co-treatment (53%) ( $p \leq 0.01$ ). In addition, cell viability was decreased to 25% upon combination therapy ( $p \leq 0.001$ ) (**Figure 3.17E**).

These results suggested that LSD1 inhibition sensitizes with Doxorubicin as supported by induced Annexin V and 7-AAD positive cells (**Figure 3.17**). On the other hand, HDAC6 inhibition with MH-25/iHDAC6 and Doxorubicin co-treatment was not synergistic however, the live-cell rate was significantly reduced to 30%. In addition to

HDAC6 inhibition, when LSD1 is further inhibited (with AD-58/iDual) the percentage of late apoptotic cells increased significantly ( $p \leq 0.001$ ) (**Figure 3.17E**).

### 3.12.2 Caspase-3/7 Staining

The mode of cellular death upon combination treatment was further examined with the activation of Caspase 3/7 as a hallmark of apoptosis [Janicke et al., 1998] and 7-AAD staining. Caspases are important proteases regulating controlled cell death and classified by their known roles in apoptosis. Caspases 8 and 9 belong to a subgroup of initiator caspases and these inactive monomers are activated by dimerization. Caspases 3, 6, and 7 belong to executioner caspases and they degrade hundreds of cellular components to induce the morphological changes for controlled cell death [McIlwain et al., 2015]. As mentioned in 3.12.1, 7-AAD was used to distinguish dead cells.

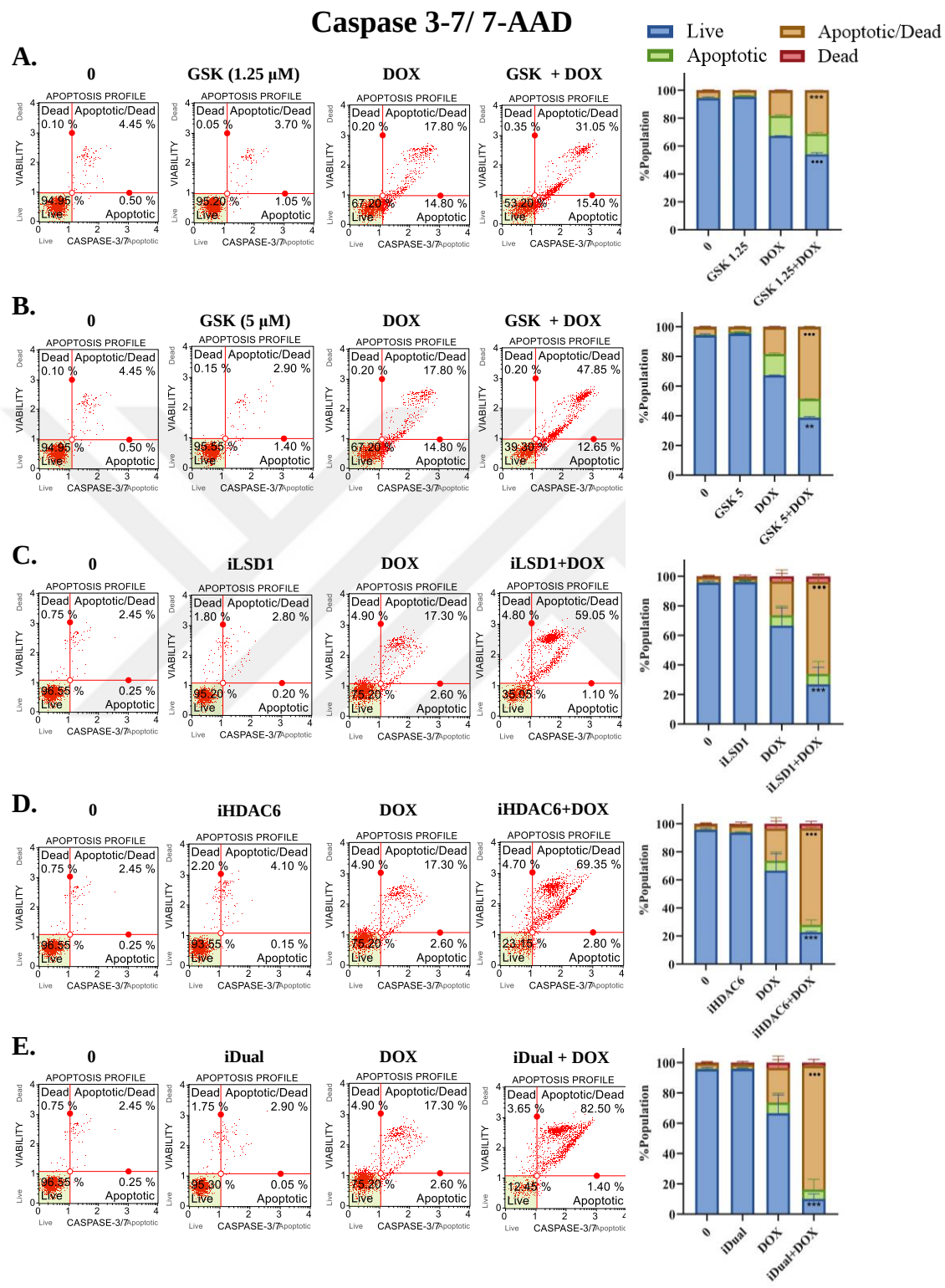


**Figure 3.18:** Flow cytometric analysis of the activated caspase-3/7 and simultaneous detection of dead cells by 7-AAD labeling

THP-1 cells were pre-treated with 1.25-5  $\mu$ M of GSK2879552 (A and B), 1.25  $\mu$ M of AD-94/iLSD1 (C), 5  $\mu$ M of MH-25/iHDAC6 (D) and 5  $\mu$ M of AD-58/iDual (E) for 72 h, then co-treated with 62.5 nM of Doxorubicin for 48h. Caspase-3/7 and 7-AAD positive cells were analyzed by flow cytometry. Data represented as the average of  $\pm$  standard deviation from 2 biologic repeats (\* $p$ <0.05, \*\* $p$ <0.01 and \*\*\* $p$ <0.001, two-way ANOVA).

Caspase-3/7(-)/7-AAD(-): Live cells; Caspase-3/7(+)/7-AAD(-): Apoptotic cells; Caspase-3/7(+)/7-AAD(+): Apoptotic/dead cells; Caspase-3/(-)/7-AAD(+): Dead cells.

As a result of flow cytometry analysis, 1.25  $\mu$ M of GSK2879552 and Doxorubicin treatment alone, induced 4, 17% of Caspase-3/7, and 7-AAD positive cells, respectively; whereas combined treatment significantly induced total apoptotic cells up to 46% ( $p \leq 0.001$ ) (**Figure 3.18A**). Consistent with Annexin V staining (**Figure 3.18B**), an increased dose of GSK2879552 (5  $\mu$ M) did not enhance Caspase 3/7 positive cells, but its combination with Doxorubicin increased total apoptotic cells up to 60% ( $p \leq 0.001$ ) (**Figure 3.18B**).

LSD1 inhibition with AD-94/iLSD1 and Doxorubicin combination, increased Caspase-3/7 and 7-AAD positive cells up to 60% ( $p \leq 0.001$ ) (**Figure 3.18C**)

HDAC6 inhibition with Doxorubicin did not result in a significant synergy in Annexin V staining (**Figure 3.23D**) however, Caspase-3/7 activation has shown a significant increase in combination therapy (70%) (**Figure 3.18D**).

Finally, dual inhibition of two epigenetic enzymes and Doxorubicin co-treatment explicitly increased Caspase-3/7 and 7-AAD positive cells. While single treatments induced 4 and 17% of apoptotic cells, this rate increased up to 82% in combinatorial treatment (**Figure 3.18E**). This data indicated that epigenetic inhibition of LSD1 or/and HDAC6 with Doxorubicin complex triggered caspase-dependent apoptotic cell death.

### **3.13 Do the Inhibitors Synergize with Clinically Used Analogues?**

In order to assess if the observed synergy with Doxorubicin is due to LSD1 inhibition in general or a phenomenon observed with our drugs only, another LSD1 inhibitor, GSK2879552 was tested. Hence, THP-1 cells were pre-treated with GSK2879552 (1.25  $\mu$ M) for 72 hours, then co-treated with Doxorubicin (62.5 nM) for 48 hours.

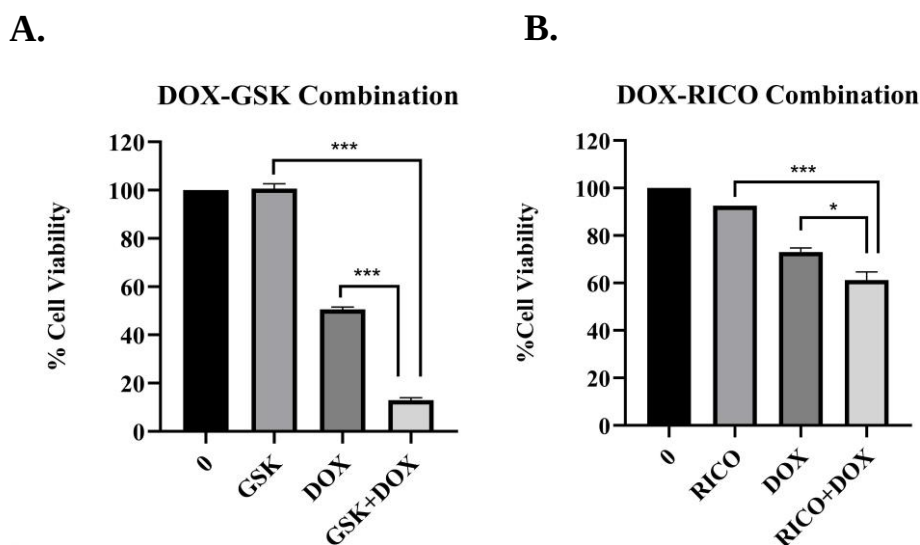


**Figure 3.19:** Cell viability in THP-1 cells after combination Doxorubicin with GSK2879552 and Ricolinostat

THP-1 cells were pre-treated with GSK2879552 (**A**) and Ricolinostat (**B**) for 72 h, then co-treated with Doxorubicin. Cell viability was measured using ATP-dependent Cell-Titer Glo assay and normalized to control cells. Data represented as the average of  $\pm$  standard deviation from 3 biological repeats (\* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ , Ordinary one-way ANOVA).

Treatment of cells with GSK2879552 alone did not reduce cell viability, while Doxorubicin treatment reduced cell viability to 50%. Interestingly, the combination of Doxorubicin and GSK2879552 significantly further reduced to cell viability to 10% ( $p \leq 0.001$ ) (**Figure 3.19A**). Therefore, our results suggested that LSD1 inhibition may be an effective mechanism to sensitize AML cells for Doxorubicin treatment.

In order to understand whether other known HDSC6 inhibitors also synergize with Doxorubicin, AML cells were co-treated with Ricolinostat. THP-1 cells were pre-treated with Ricolinostat (0.62  $\mu$ M) for 72 hours, then co-treated with Doxorubicin (62.5 nM) for 48 (**Figure 3.19B**). Similar to our previous experiments, doses at which single drug treatment does not lead to cell death above 30%, were selected to study synergy. As expected, HDAC6 inhibition with Ricolinostat alone or Doxorubicin alone did not induce critical cell death, but their combination reduced cell viability to 60% suggesting additive and synergistic effect (**Figure 3.19B**).

### 3.14 How Does the Synergy Work?

Doxorubicin is a well-studied anticancer drug and is known to induce its toxicity through three separate mechanisms: 1- inhibition of Topoisomerase II, leading to double-strand DNA breaks, 2- induction of reactive oxygen species damaging cellular macromolecules and 3- through induction of DNA damage via DNA intercalation [Thorn et al., 2011]. In order to assess if anyone of the toxic effects of Doxorubicin is enhanced via LSD1 and/or HDAC6 inhibition, we dissected the roles of Doxorubicin and studied separately.

#### 3.14.1 Assessment of Synergy through Topoisomerase II Inhibition Function

##### 3.14.1.a Etoposide

We hypothesized that if inhibition of Topoisomerase II is essential for DOX-induced synergy, then another Topo II inhibitor should also synergize with our compounds. To test this idea, we used Etoposide, which is a known, clinically used Topo II inhibitor. THP-1 cells were pre-treated with epigenetic inhibitors (1.25  $\mu$ M iLSD1, 5  $\mu$ M iHDAC6 and 5  $\mu$ M iDual) for 72 hours and co-treated with Etoposide (0.2  $\mu$ M) for 48 hours. We observed that neither the single treatment of the drugs alone nor the combination resulted in a significant decrease in cell viability in any of the doses that we tested (data not shown) suggesting that the synergy is not likely through enhanced Topo II inhibition (**Figure 3.20**).

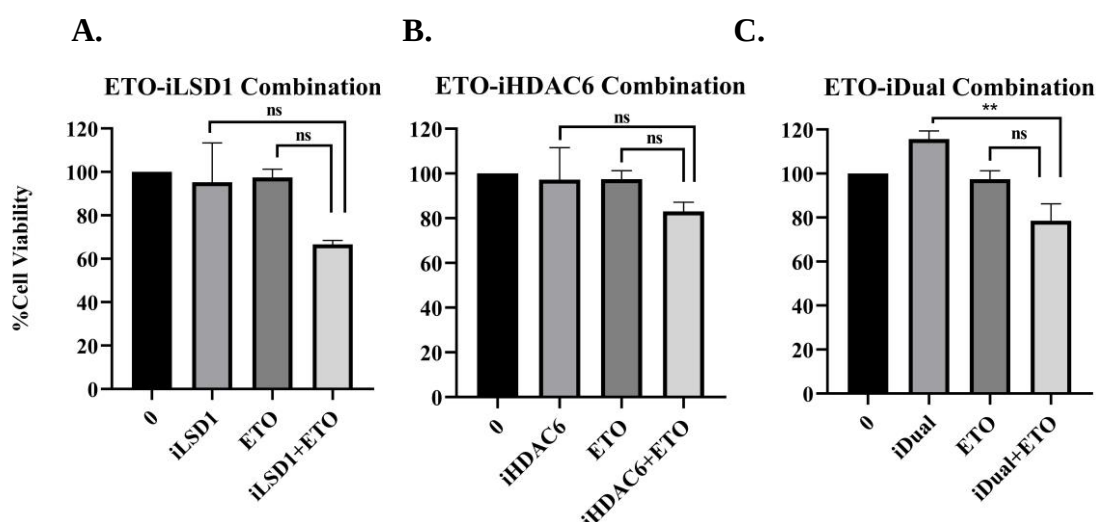


**Figure 3.20:** Cell viability in THP-1 cells combined with Etoposide and novel inhibitors

THP-1 cells were pre-treated with AD-94/iLSD1(**A**), MH-25/iHDAC6 (**B**), and AD-58/iDual (**C**) for 72 h, then co-treated with Etoposide. Cell viability was measured using ATP-dependent Cell-Titer Glo assay and normalized to control cells. Data represented as the average of  $\pm$  standard deviation from 3 biological repeats (\* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ , Ordinary one-way ANOVA).

#### 3.14.1.b $\gamma$ H2AX Activation

Topoisomerase II inhibition with Doxorubicin is known to lead double-strand breaks on DNA [Duran et al., 1996]. Following DNA damage, cells enter an intense DNA repair mechanism and whose DNA that cannot be repaired eventually, dies. Double-stranded breaks (DSBs) generate rapid accumulation in the phosphorylation of histone H2A variant H2AX [Sharma et al., 2012]. We further investigated the role of double-strand DNA breaks upon combination therapy with Doxorubicin. In order to evaluate the sensitivity of AML cells to DSBs, cells were pre-treated with epigenetic inhibitors for 72 hours (1.25  $\mu$ M AD-94/iLSD1, 5  $\mu$ M MH-25/iHDAC6, and 5  $\mu$ M AD-58/iDual) and co-treated with Doxorubicin (62.5 nM) for 6 hours. The expressions of  $\gamma$ H2AX upon treatments were quantitatively evaluated using flow cytometry (**Figure 3.21**).

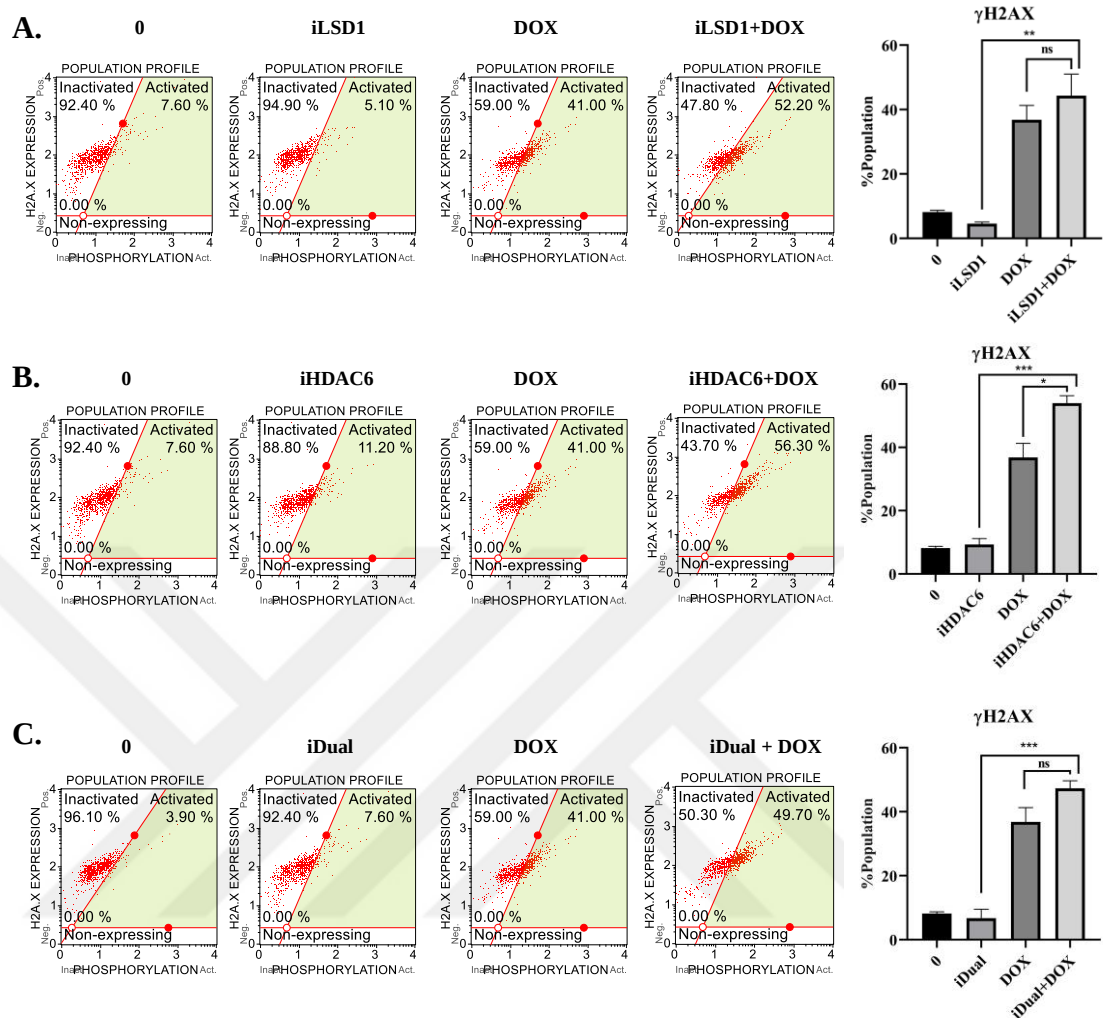


**Figure 3.21:** Flow cytometric analysis of the  $\gamma$ H2AX positive cells

THP-1 cells were pre-treated with AD-94/iLSD1 (A), MH-25/iHDAC6 (B), and AD-58/iDual (C) for 72 h, then co-treated with 62.5 nM of Doxorubicin for 6 h. H2AX activated cells were analyzed by flow cytometry. Data represented as the average of  $\pm$  standard deviation from 2 biologic repeats (\* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ , Ordinary one-way ANOVA).

The inactivated quadrant indicates the cells expressing H2AX without phosphorylation and the activated quadrant indicates the  $\gamma$ H2AX phosphorylated cells.

Doxorubicin treatment for 6 hours appeared to induce DSBs up to 40% (Figure 3.21) and 12 hours of treatments induced >80% DSB in THP-1 cell (data not shown). In Doxorubicin alone treatment, cells remained viable after 48 hours at the same dose, thus this data suggested that without epigenetic inhibitor the DNA damage must have been repaired.

HDAC6 inhibition with Doxorubicin significantly induced  $\gamma$ H2AX activation in THP-1 cells ( $p \leq 0.05$ ) (**Figure 3.21B**). Treatments with AD-94/iLSD1 and AD-58/iDual did not increase H2AX phosphorylation, and no significant increase was observed in their combination with Doxorubicin (**Figure 3.21A, Figure 3.21C**). The additive anti-cancer effect in response to HDAC6 inhibition with Doxorubicin may be due to the increase in DSBs. However, LSD1 inhibition did not lead to further H2AX-dependent cell death.

Overall, these results suggested that epigenetic drug sensitization does not occur through TopoII inhibition or  $\gamma$ H2AX activation.

### 3.14.2 ROS Generation Profile upon Combination Treatment

Anti-cancer agents such as Doxorubicin lead to overproduction of reactive oxygen species (ROS) [Cappetta et al., 2017]. Free radical generation can create a nick in DNA and can damage essential enzymes and structural proteins. DNA damage by ROS may lead to multiple lesions, modified bases, and double-strand breaks. In cancer cells, several repair enzymes maintain cell viability by reverting the damage. However, when the damage reaches a certain point, cell death becomes inevitable. In order to understand how the inhibitors, sensitize AML cells for Doxorubicin treatment, the contribution of inhibitors to ROS profiles in THP-1 cells upon combination therapy was quantitatively investigated using flow cytometry (**Figure 3.22**). For evaluation of ROS generation, cells were pre-treated with epigenetic inhibitors (1.25  $\mu$ M AD-94/iLSD1, 5  $\mu$ M MH-25/iHDAC6, and 5  $\mu$ M AD-58/iDual) for 72 hours and co-treated with Doxorubicin (62.5nM) for 24 hours and total ROS was measured via flow cytometry.

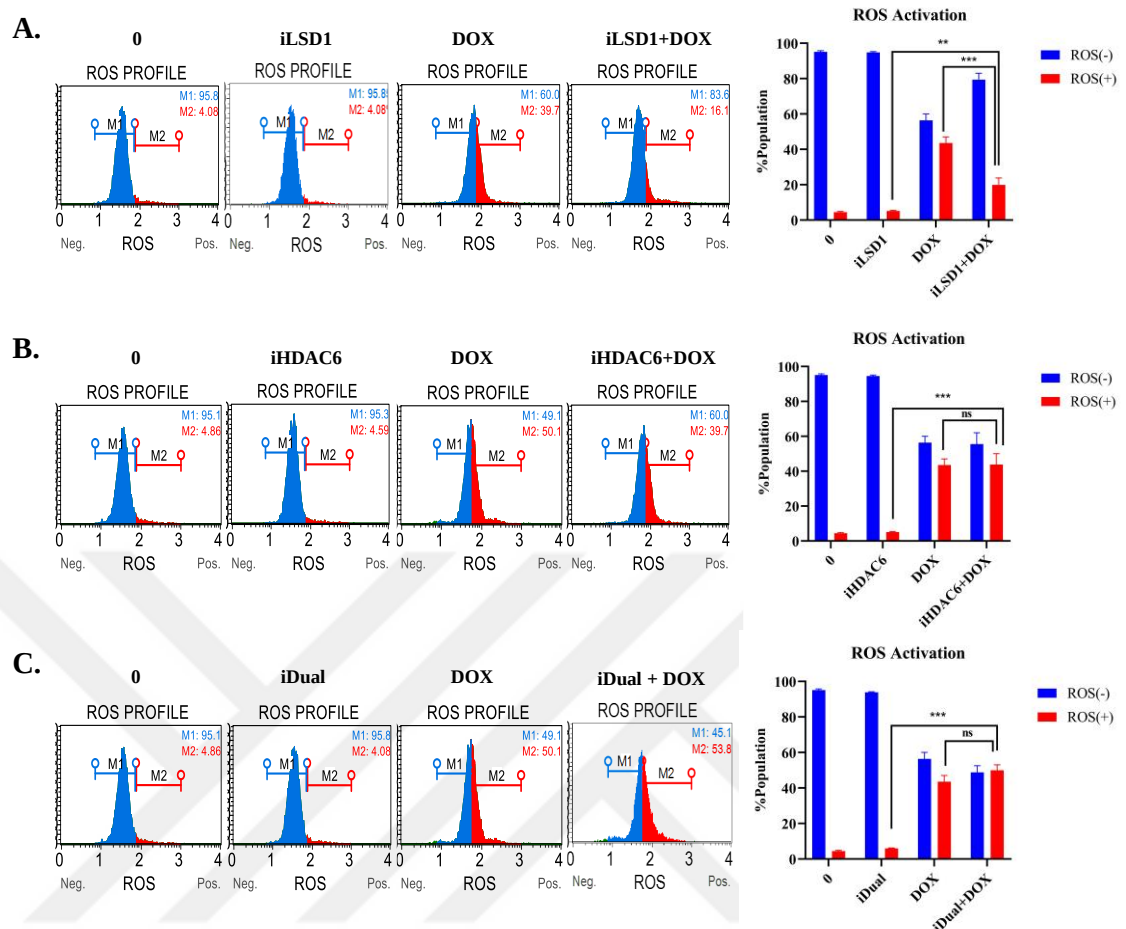


**Figure 3.22:** Ros generation profile upon treatment in THP-1 cells

THP-1 cells were pre-treated with AD-94/iLSD1 (**A**), MH-25/iHDAC6 (**B**), and AD-58/iDual (**C**) for 72 h, then co-treated with 62.5 nM of Doxorubicin for 24 h. Oxidative stress generation in THP-1 cells was assessed by flow cytometry. Data represented as the average of  $\pm$  standard deviation from 2 biological repeats (\* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ , Ordinary one-way ANOVA).

M2 population indicates ROS (+) cells.

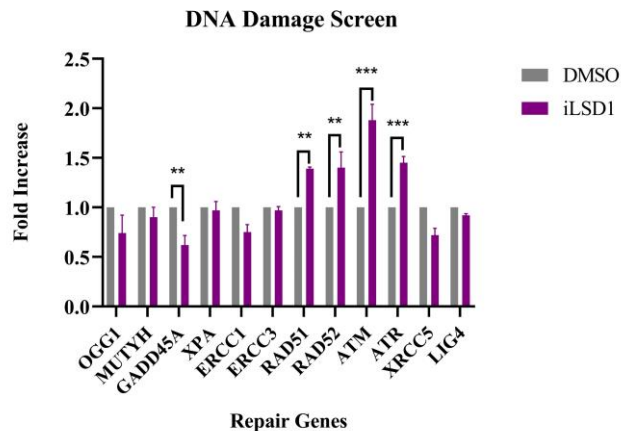
While inhibition of LSD1 or HDAC6 with our epigenetic drugs did not increase oxidative stress, Doxorubicin-induced formation of ROS, as described in the literature. Contrary to our expectations, the combination significantly reduced oxidative stress down to 16% ( $p \leq 0.01$ ) suggesting that epigenetic drugs may reduce the ROS load in cell (**Figure 3.22A**). On the other hand, iHDAC6 or iDual treatment did not contribute any significant increase in the Doxorubicin effect (**Figure 3.22B**, **Figure 3.22C**). This data revealed that the activated cell death upon combination was not through ROS activation.

### 3.14.3 Assessment of Screening DNA Damage Response Following Inhibitor Treatment

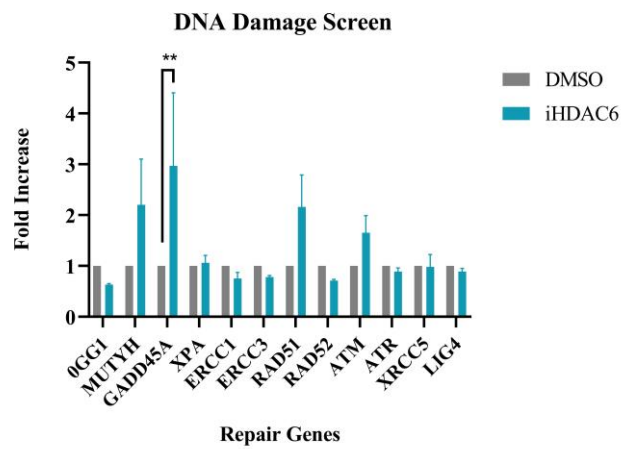
Doxorubicin intercalates into DNA strands and creates DNA damage. Different repaired mechanisms are known to be activated upon DNA damage.

Cells have different repair mechanisms, which get activated upon different types of DNA damage. DNA damage due to oxidation, deamination, or alkylation distort the DNA helix structure and are corrected by base excision repair (BER) [Krokan and Bjoras, 2013]. DNA damage induced by UV irradiation, mutagens, and usage of chemotherapeutic agents is removed by nucleotide excision repair (NER) [Scharer, 2013]. Homology directed repair (HDR) and non-homologous ends joining (NHEJ) processes are activated upon double-strand breaks [Zaboikin et al., 2017]. We hypothesized that one of the mechanisms of synergy with Doxorubicin may be due to the sensitization of cells with epigenetic drugs via reduced DNA damage response. To test our hypothesis, the change in the expression levels of key DNA repair genes of each pathway described above was examined in AML cells. THP-1 cells were treated with epigenetic inhibitors (1.25  $\mu$ M AD/-94/iLSD1, 5  $\mu$ M MH-25/iHDAC6, and 5  $\mu$ M AD-58/iDual) for 72 hours. The expression of selected DNA repair genes was evaluated with RT-qPCR (**Figure 3.23**).

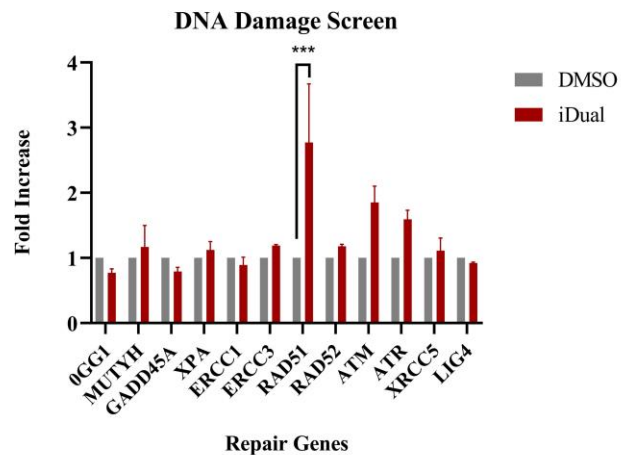
A.



B.



C.



D.

| Repair Pathway              | Genes                  |
|-----------------------------|------------------------|
| Base Excision Repair        | OGG1, MUTYH, GADD45A   |
| Nucleotide Excision Repair  | XPA, ERCC1, ERCC3      |
| Homology Directed Repair    | RAD51, RAD52, ATM, ATR |
| Non-Homologous Ends Joining | XRCC5, LIG4            |

**Figure 3.23:** DNA damage repair gene expressions upon inhibitor treatment

RT-qPCR analysis of DNA repair genes upon AD-94/iLSD1 (**A**), MH-25/iHDAC6 (**B**), and AD-58/iDual (**C**) for 72 h in THP-1 cells. Table showing repair pathways and related genes (**D**). Gene expression was normalized to two housekeeping genes,  $\beta$ -actin, and GAPDH. Relative expression was determined with the  $2^{(-\Delta\Delta CT)}$  method. Data represented as the average of  $\pm$  standard error of the mean of 3 biological repeats with duplicates (\* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ , two-way ANOVA).

RT-qPCR analysis of the present study demonstrated that AD-94/iLSD1 significantly increased homology-directed repair-related genes RAD51, RAD52, ATM, and ATR (**Figure 3.23A**). This data suggesting that DSB repair mechanisms were already induced in AD-94/iLSD1 treated cells before Doxorubicin exposure.

Histone Deacetylase inhibitors are known to induce growth arrest and lead differentiation of apoptosis in tumor cells [Hirose et al., 2003]. Stressful growth arrest conditions and DNA-damaging agents are known to increase GADD45A transcription. Similarly, HDAC6 inhibition significantly increased GADD45A expression in AML cells ( $p \leq 0.01$ ) (**Figure 3.23B**). In addition, AD-58/iDual treatment only led to a significant increase in the RAD51 gene ( $p \leq 0.001$ ) (**Figure 3.23C**). Overall, this data demonstrated contrary to our expectations the inhibitors activated that repair gene at least for the ones that were under study. Therefore, the sensitization of AML cells for Doxorubicin treatment still needs to be discovered further, which will be discussed in the next section.

### 3.15 RNA-seq Analysis

In order to understand the molecular chain of events bearing MLL-AF9 fusion in response to inhibition of LSD1, HDAC6, or both in cells, we performed RNA seq analysis and examined which genes were differentially expressed following drug treatment. We also dug further for genes that may explain how these inhibitors might synergize with Doxorubicin. THP-1 cells were treated with epigenetic inhibitors for 72 hours, then RNA sequencing was performed for a detailed examination of differentially expressed genes upon LSD1 and HDAC6 inhibition.

The transcriptomic differences of each sample between the treatment groups were shown with Multidimensional scaling (MDS) plots (**Figure 3.24**). In this plot, the samples showing similar transcriptomic patterns localize close to each other, while distance increases as the differences grow. As shown in **Figure 3.24**, vehicle and negative control MH-18 samples localized very close to each other, which strongly suggests that the

negative inhibitor does not target a specific enzyme in AML cells, since there is no significant difference in the expression pattern compared to mock-treated controls, as expected. This data was also correlated with the number of differentially expressed genes (DEGs) (**Figure 3.25**). On the other hand, in negative control-treated AML cells, there was no significantly expressed gene (highest and lowest logFC's were -0.9 and 0.9).

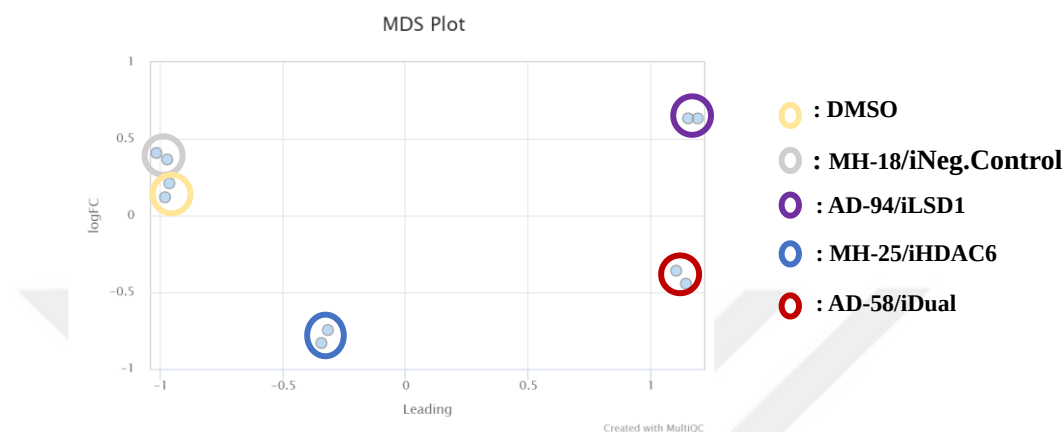
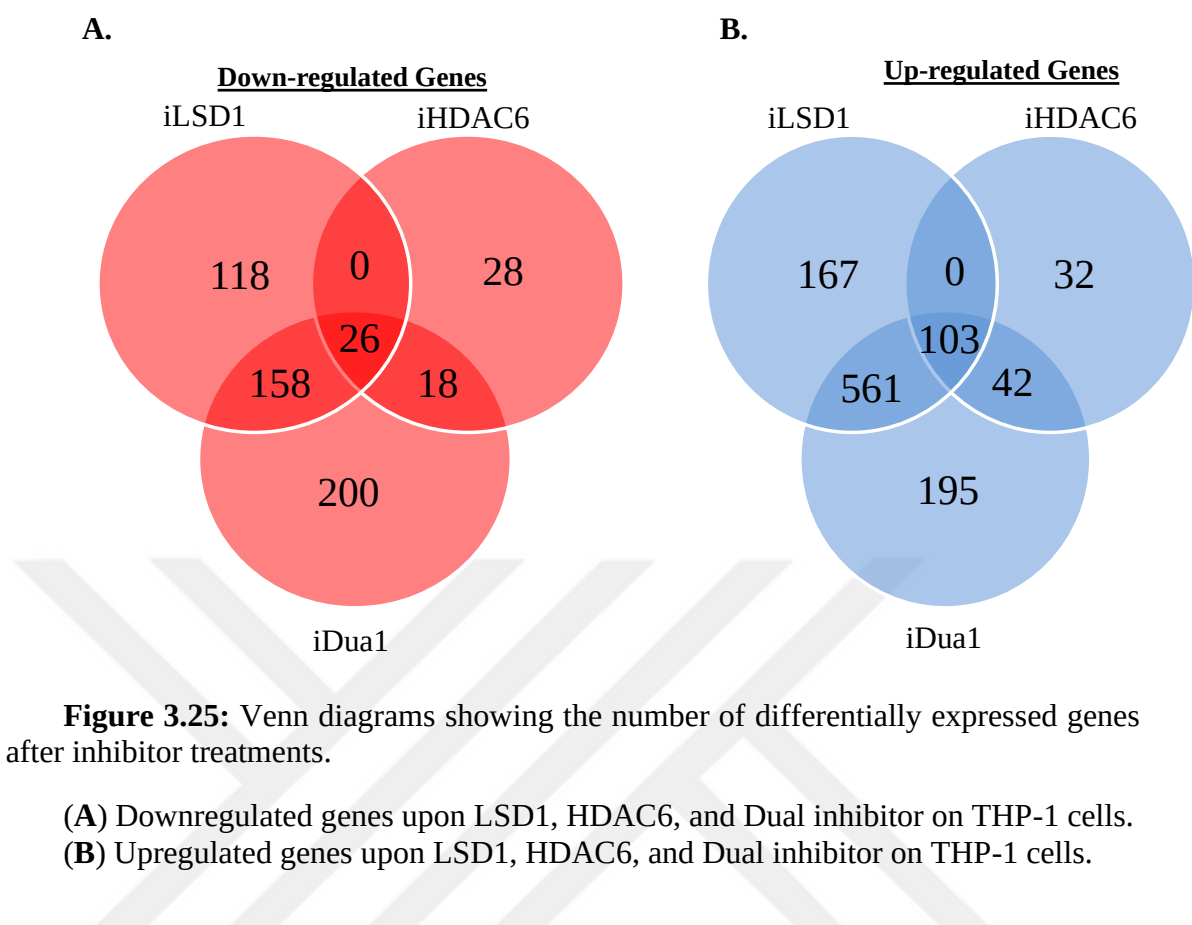


**Figure 3.24:** MDS plot reflecting transcriptomic similarity after different treatment groups.

This figure was created by Dr. Hamza Syed, by using MultiQC software.

As shown in the Venn Diagrams (**Figure 3.25**), the number of up-regulated genes was higher than the down-regulated genes on the inhibitor groups (Differentially Expressed Genes (DEGs) with  $\logFC < -1$  and  $\logFC > 1$  were considered as significant). LSD1 inhibition led to a down-regulation of 302 and up-regulation of 831 genes. On the other hand, HDAC6 inhibition resulted in 72 down-regulated and 177 up-regulated genes. Finally, AD-58/Dual inhibitor resulted in 402 down-regulated and 899 up-regulated genes that passed the threshold of significance ( $p < 2.511301e-06$ ). 26 genes were downregulated and 103 genes were upregulated in all-drug treated samples except for MH-18/iNeg.Cntrl. As indicated in the Venn Diagrams, AD-94/iLSD1 and MH-25/iHDAC6 did not share a common gene. Finally, AD-58/iDual contained several common genes with LSD1 and HDAC6 inhibitor (**Figure 3.25**), supporting that they share common target enzymes.



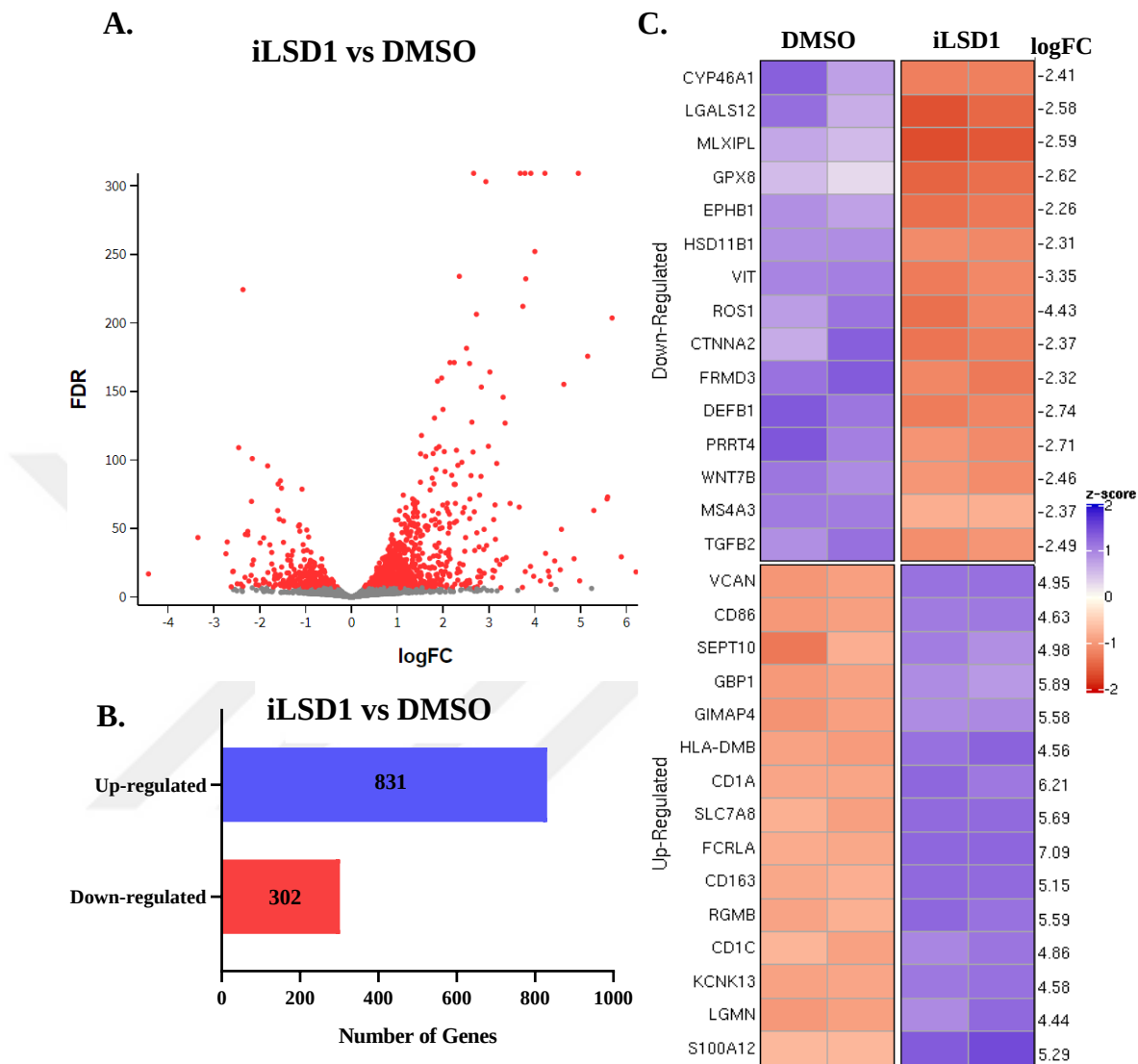


**Figure 3.26:** Differentially expressed genes upon LSD1 inhibition with AD-94 treatment.

(A) Volcano plot of differentially expressed genes upon AD-94 treatment. (B) The number of up/down-regulated genes. (C) Heat map showing differentially expressed top 30 genes upon LSD1 inhibition with AD-94 treatment. Genes were selected by their z-score (-2, 2). Volcano plot and the heat maps were created by Dr. Hamza Syed by using MultiQC software. Blue: Upregulated genes, Red: Downregulated genes.

Consistent with our earlier results (Figure 3.14), the hierarchical clustering analysis of the genes with the greatest differential response to AD-94/iLSD1 treatment indicated that the monocyte-macrophage differentiation-related genes (*ANXA2*, *MPEG1*, *CD163*), were up-regulated (Figure 3.26). Accordingly, AD-94/iLSD1 treatment resulted in the upregulation/downregulation of DEG in response to LSD1 inhibition (*AIM1*, *ANXA2*,

*ARHGAP26, CAMSAP2, CCDC109B, CD-53, DTX4, FCRLA, GLIPR1, GPR183, HEXB, HIPK2, IL10RA, IQGAP2, KCTD12, KIAA1244, KIF13A, KIF26B, LPXN, MPEG1, OSBP1L, PLBD1, RGMB, RNF126P1, S100A12, SASH1, SCPEP1, SGMS2, SH3BP5, SLC7A7, SLC15A3, SLC37A2, SNX7, TLR8, TMEM243, TRIM2 and LY96*) also consistent with the literature (**Table 3.3**). These results indicate that AD-94 inhibits LSD1 and this inhibition results in the upregulation of monocyte-macrophage differentiation genes on AML cells.

**Table 3.3:** Table showing differentially expressed LSD1-related genes, upon AD-94 treatment.

The table includes logFC, description of the gene, and correlation with literature by the PMID of the relevant article.



**DEG's of LSD1-Related Genes**

| Gene Name | logFC | Description                                                                                            | Literature/<br>PMID                                                               |
|-----------|-------|--------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------|
| ANXA2     | 2.35  | Annexin A2 Calcium-regulated membrane-binding protein                                                  |                                                                                   |
| CAMSAP2   | 1.61  | Calmodulin Regulated Spectrin Associated Protein Family Member 2                                       |                                                                                   |
| CD53      | 2.15  | Leukocyte surface antigen CD53 Cell Surface Glycoprotein                                               |                                                                                   |
| FCRLA     | 7.09  | Fc receptor is a protein found on the surface of certain cells – including, macrophages                |                                                                                   |
| HEXB      | 1.51  | Hexosaminidase Subunit Beta Hexosaminidase B                                                           |                                                                                   |
| KIF26B    | 1.82  | Intracellular Motor Protein                                                                            | Similarly specific LSD1 inhibitor 0RY-1001 increased expression of the same genes |
| LPXN      | 2.04  | Leupaxin preferentially expressed in hematopoietic cells                                               |                                                                                   |
| OSBP11L   | 2.63  | Oxysterol-binding protein-related protein 11                                                           |                                                                                   |
| S100A12   | 5.29  | S100 family of proteins containing 2 EF-hand calcium-binding motifs,                                   |                                                                                   |
| SCPEP1    | 1.43  | Serine Carboxypeptidase 1                                                                              |                                                                                   |
| SH3BP5    | 1.81  | B-cell SH3 Domain Binding Protein 5                                                                    | (29502954)                                                                        |
| SLC7A7    | 1.4   | Solute Carrier Family 7 Member 7                                                                       |                                                                                   |
| SNX7      | 1.48  | Sorting Nexin 7                                                                                        |                                                                                   |
| TMEM243   | 2.43  | Transmembrane Protein 243                                                                              |                                                                                   |
| ARHGAP26  | 1.19  | Rho GTPase Activating Protein 26                                                                       |                                                                                   |
| CCDC109B  | 3.35  | Coiled-coil domain containing 109B is a potential calcium uniporter protein                            |                                                                                   |
| DTX4      | 3.8   | 3 ubiquitin-protein ligase DTX4                                                                        |                                                                                   |
| GPR183    | 2.65  | G-protein coupled receptors-183                                                                        |                                                                                   |
| KIAA1244  | 2.08  | Regulator of ARF Protein Signal Transduction                                                           |                                                                                   |
| KIF13A    | 2.04  | Kinesin Member Localized on the Endosomes                                                              |                                                                                   |
| MPEG1     | 4.22  | Macrophage-expressed gene 1 protein                                                                    |                                                                                   |
| RGMB      | 5.59  | RGMB is a glycosylphosphatidylinositol (GPI)-anchored member of the repulsive guidance molecule family | 31537694                                                                          |
| RNF126P1  | 2.58  | Ring Finger Protein 126 Pseudogene 1                                                                   |                                                                                   |
| SASH1     | 1.22  | SAM And SH3 Domain Containing 1                                                                        |                                                                                   |
| SGMS2     | 2.32  | Sphingomyelin                                                                                          |                                                                                   |
| SLC15A3   | 1.90  | Solute Carrier Family 15 Member 3                                                                      |                                                                                   |
| SLC37A2   | 2.26  | Solute Carrier Family 37 Member 2                                                                      |                                                                                   |
| TRIM2     | 2.14  | Tripartite Motif Member                                                                                |                                                                                   |
| TLR8      | 2.49  | Toll-like Receptor                                                                                     |                                                                                   |
| AIM1      | 2.73  | Absent in Melanoma 1 gene                                                                              | 23072722                                                                          |
| GLIPR1    | 2.58  | GLI Pathogenesis Related 1                                                                             | 29633255                                                                          |
| HIPK2     | 1.77  | Homeodomain Interacting Protein Kinase 2                                                               | 29156705                                                                          |
| IQGAP2    | 1.55  | IQ Motif Containing GTPase Activating Protein 2 Ligand protein of LSD1                                 | 29656462                                                                          |
| KCTD12    | 2.93  | potassium channel tetramerization domain                                                               | 32003940                                                                          |
| LY96      | 4.24  | LY96 (Lymphocyte Antigen 96)                                                                           | 26837761                                                                          |

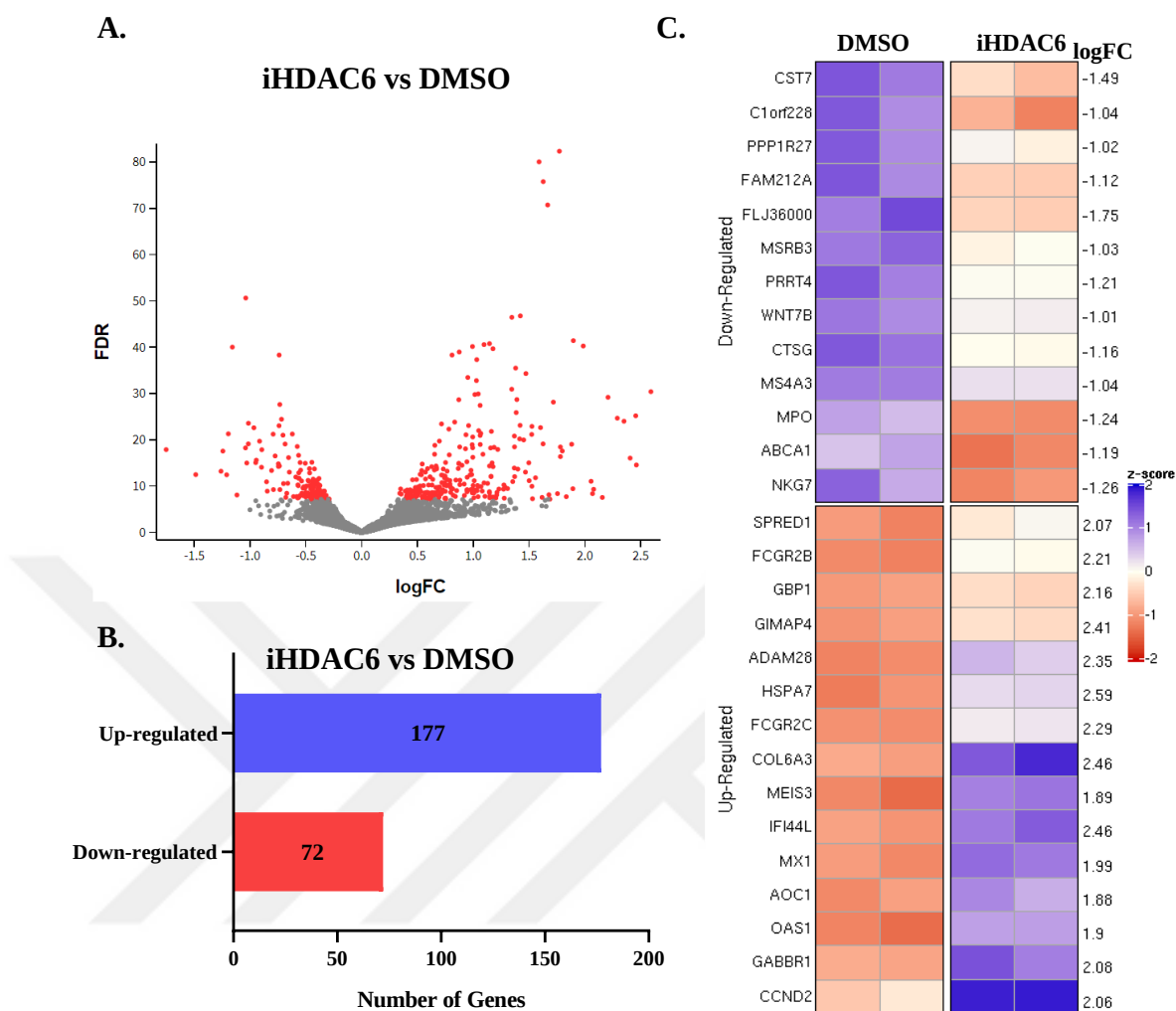


**Figure 3.27:** Differentially expressed genes upon HDAC6 inhibition with MH-25 treatment.

(A) Volcano plot of differentially expressed genes upon MH-25 treatment. (B) The number of up/down-regulated genes. (C) Heat map showing differentially expressed top 30 genes upon HDAC6 inhibition with MH-25 treatment. Genes were selected by their z-score (-2, 2). Volcano plot and the heat maps were created by Dr. Hamza Syed by using MultiQC software. Blue: Upregulated genes, Red: Downregulated genes.

Differentially expressed gene analysis revealed that the HDAC6-related genes (*DDIT4*, *OAS1*, *OAS2*, *OAS3*) were up-regulated in MH-25 treated samples when compared with DMSO (Figure 3.27). Additionally, as shown in Table 3.4, MH-25 treatment led the upregulation of other HDAC6-related genes (*FNBP1L*, *IFI44*) also correlated with the literature (Table 3.4).

**Table 3.4:** Table showing differentially expressed HDAC6-related genes, upon MH-25 treatment.

The table includes logFC, description of the gene, and correlation with literature by the PMID of the relevant article.

| <u>DEG's of HDAC6 Related Genes</u> |       |                                                                                                                                                                             |          |
|-------------------------------------|-------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------|
| Gene Name                           | logFC | Description                                                                                                                                                                 | PMID     |
| DDIT4                               | 1.42  | DNA Damage Inducible Transcript 4, HDAC6 inhibition induces the expression of DDIT4                                                                                         | 21037108 |
| FBNP1L                              | 1.06  | Formin-binding protein 1-like, coordinate membrane tubulation with reorganization of the actin cytoskeleton during endocytosis<br>FBNP1L is increased upon HDAC6 inhibition | 22117042 |
| HSP7A                               | 2.35  | HSF1 Transcription Factor Regulates HSP7A and affects HDAC6 interaction                                                                                                     | 28369544 |
| IFI44                               | 1.72  | Interferon Induced Protein 44, IFI44 expression increases after HDAC6 inhibition.                                                                                           | 26759180 |
| RASGRP2                             | -0.97 | RAS guanyl releasing protein 2, HDAC inhibitors degrade RASGRP family                                                                                                       | 27890930 |

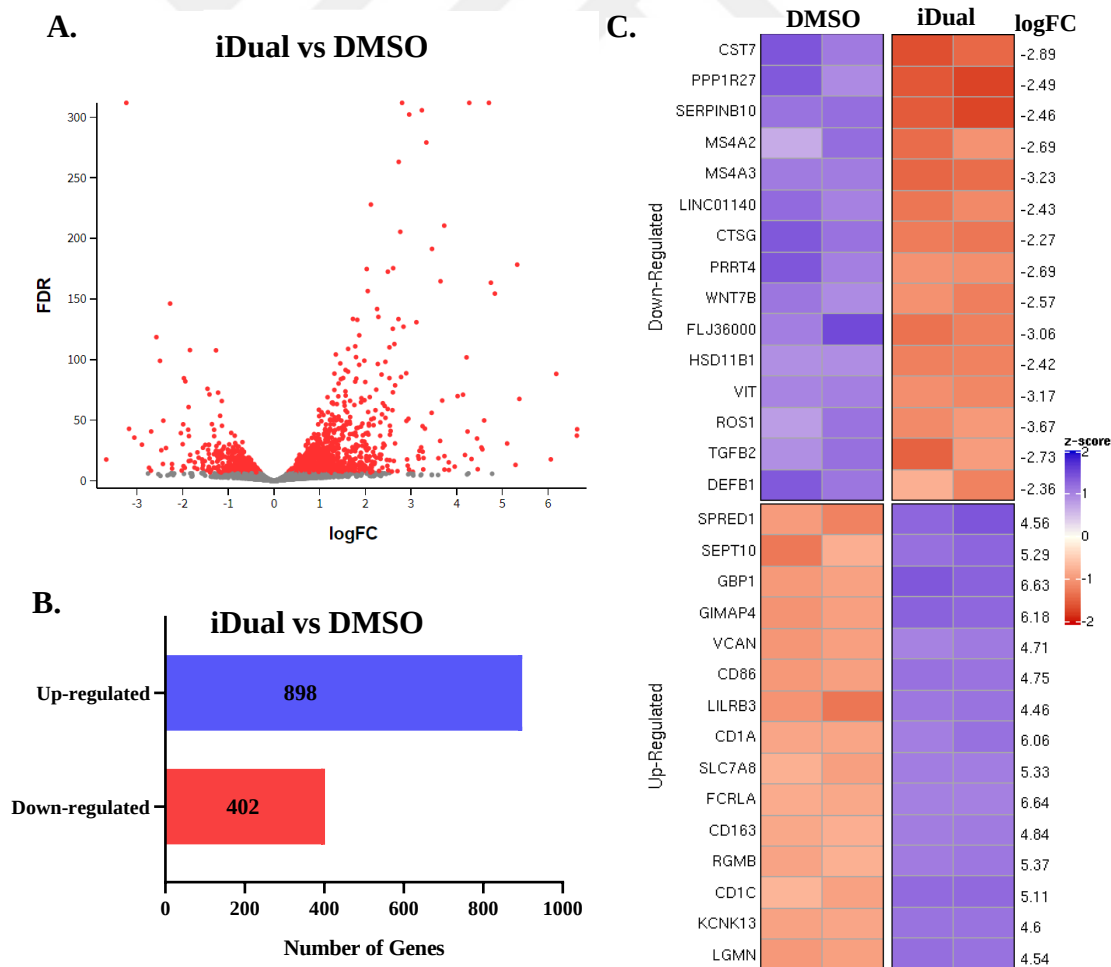


**Figure 3.28:** Differentially expressed genes upon LSD1/HDAC6 inhibition with AD-58 treatment.

(A) Volcano plot of differentially expressed genes upon AD-58 treatment. (B) The number of up/down-regulated genes. (C) Heat map showing differentially expressed top 30 genes upon LSD1/HDAC6 inhibition with AD-58 treatment. Genes were selected by their z-score (-2, 2). Volcano plots and heat maps are created by Dr. Hamza Syed by using MultiQC software. Blue: Upregulated genes, Red: Downregulated genes.

The heat map with the top 20 DEG's showed that LSD1-related genes (*ANXA2*, *MPEG1*, *CD163*, *KCTD12*, *AIM1*, *CD53*, *ITGAM*, *CD86*, *GLIPR1*, *CD163*, and *DTX4*) were up-regulated in iDual treated samples when compared with DMSO (**Figure 3.28**). Similar with AD-94/iLSD1 treatment, AD-58/iDual resulted in the upregulation of monocyte-macrophage differentiation-related genes (*ANXA2*, *CD14*, *CD163*, *CD44*, *CLEC5A*, *c-MAF*, *CSFR1*, *GBP2*, *HLA-DMA*, *HLA-DMB*, *HLA-DPA1*, *IFNGR1*, *ITGB7*, *JUN*, *LRP1*, *MPEG1*, *MS4A4A*, *PRNT3*) again consistent with the literature verifying the inhibitors actually engage with and inhibit their intended targets (**Table 3.5**). These results showed that in the terms of DEG's AD-94/iLSD1 and AD-58/iDual shared a higher number of common genes (664 up-regulated, 184 down-regulated), whereas MH-25/iHDAC6 and AD-58/iDual shared fewer common genes as indicated in Table 3.4.

**Table 3.5:** Table showing differentially expressed monocyte-macrophage differentiation related-genes, upon both AD-94 and AD-58 treatment.

The table includes logFC, description of the gene, and correlation with literature by the PMID of the relevant article.

**DEG's of Monocyte-Macrophage Differentiation Related Genes upon both AD-94 and AD-58 Treatment**

| Gene Name        | logFC          | Description                                                                             | PMID                 |
|------------------|----------------|-----------------------------------------------------------------------------------------|----------------------|
| ANXA2            | 2.35           | Activated during Monocyte Macrophage differentiation                                    | 29502954             |
| CD14             | 2.36           | Human Protein Synthesized by Macrophages                                                | 24312856             |
| CD163            | 5.15           | Marker of Cells from the Monocyte/macrophage Lineage                                    | 23738227             |
| CD44             | 1.34           | Cell surface marker involved in monocyte differentiation                                | 29196459             |
| CLEC5A           | 2.61           | Macrophage c-type lection receptor                                                      | 29126450             |
| c-MAF            | 3.66           | Essential Transcription Factor for IL-10 Gene Expression in Macrophages                 | 15749884             |
| CSF1R            | 1.51           | Macrophage colony-stimulating factor 1                                                  | 25368618             |
| FPR1             | 3.14           | Expressed in the cell surface of resting macrophages                                    | Gemperle et al. 2012 |
| GBP2             | 3.13           | Highly activated by the interferon-gamma during macrophages activation                  | 17266443             |
| HLA-DMA/DMB/DPA1 | 2.47/4.55/2.32 | These Genes Belongs to the HLA Class II Alpha Chain Paralogues Expressed in Macrophages | 24349126             |
| IFNGR1           | 1.25           | Encodes for gamma interferon receptor on macrophages                                    | 31585982             |
| ITGB7            | 1.83           | Macrophage specific integrin                                                            | 27313577             |
| JUN              | 1.82           | Transcription factor regulates macrophage activation                                    | 1712226              |
| LRP1             | 2.24           | Modulates macrophage homeostasis                                                        | 29144234             |
| MPEG1            | 4.22           | Macrophage-expressed gene 1 protein                                                     | 21084707             |
| MS4A4A           | 1.90           | A novel cell surface marker for M2 macrophages                                          | 28303902             |
| PRTN3            | -2.16          | Expressed on the cell surface of monocytes                                              | 330392974            |

A final question was how these inhibitors sensitize AML cells for Doxorubicin treatment? In order to understand that, DEGs were further analyzed in the literature according to their relationship with Doxorubicin. AD-94 and AD-58 resulted in the upregulation of *LRP1*, *AGPAT9*, *BTG1*, and *HIPK2* genes which known to increase sensitivity against Doxorubicin. Similarly, HDAC6 inhibitor MH-25 increased *IFI44L*, which also known to be the overexpression of the gene decreases chemoresistance towards Doxorubicin (**Table 3.6**). Among these genes, ALOX5 appears as a considerable target with increased expression in LSD1, HDAC6, and their dual inhibition. ALOX5 is reported to function as a tumor-suppressor and it shows increased sensitivity against chemotherapy agents such as Doxorubicin on AML cells [Jiang et al.2016; Wang et al., 2017].

**Table 3.6:** Table showing differentially expressed Doxorubicin sensitizer-genes, upon AD-94, MH-25, and AD-58 treatment.

The table includes logFC, description of the gene, and correlation with literature by the PMID of the relevant article.

**Differentially Expressed Doxorubicin Sensitizer Genes upon LSD1&HDAC6 Inhibition**

| Gene Name | logFC | Description                                                                                                                                               | PMID     |
|-----------|-------|-----------------------------------------------------------------------------------------------------------------------------------------------------------|----------|
| AGPAT9    | 1.82  | Increased AGPAT9 expression in MCF-7 cells was reported to increase the chemosensitivity to doxorubicin                                                   | 26110566 |
| ALOX5     | 2.27  | ALOX5 exhibits anti-tumor and Doxorubicin-sensitizing effects in MLL-rearranged leukemia                                                                  | 28500307 |
| BTG1      | 1.72  | Doxorubicin-induced cell death is mediated by BTG family                                                                                                  | 18840609 |
| HIPK2     | 1.77  | HIPK2 regulates chemosensitivity including Doxorubicin                                                                                                    | 29434701 |
| IFI44L    | 2.46  | Overexpression of IFI44L decreased chemoresistance towards Doxorubicin                                                                                    | 29848298 |
| LRP1      | 2.24  | Increased LRP1 may be associated with higher endocytosis of upregulated transporter proteins at the cell surface, thus increased Doxorubicin accumulation | 29644529 |
| WNT7B     | -2.45 | Wnt7B Inhibits Osteosarcoma Cell Growth, Migration, Invasion, and Sensitivity to Doxorubicin                                                              | 31601147 |

**Table 3.7:** Table showing other differentially expressed gene families, upon AD-94, MH-25, and AD-58 treatment.

The table includes regulation and description of the relevant gene.

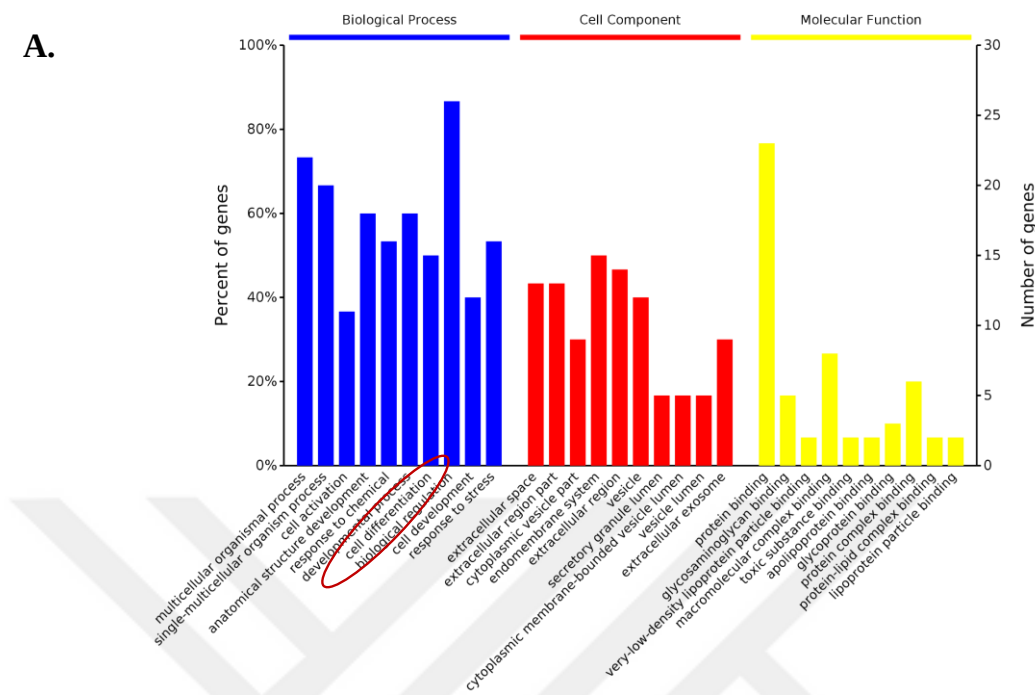
**Other Groups of Differentially Expressed Gene Families**

| Gene Name | Regulation  | Treatment     | Description                                                                                                                                                                               |
|-----------|-------------|---------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| CTSB      | Upregulated | AD-94 / AD-58 | Cathepsins are a enzyme family of proteases. Generally, cathepsins are activated in lysosomes at lower pH                                                                                 |
| CTSH      | Upregulated | AD-94 / AD-58 |                                                                                                                                                                                           |
| CTSZ      | Upregulated | AD-94 / AD-58 |                                                                                                                                                                                           |
| S100A4    | Upregulated | AD-94 / AD-58 | S100 Ca <sup>2+</sup> -binding proteins are associated with regulation of the cell cycle, cell differentiation, and apoptosis, transduction of intracellular Ca <sup>2+</sup> signals     |
| S100A10   | Upregulated | AD-94 / AD-58 |                                                                                                                                                                                           |
| S100A12   | Upregulated | AD-94 / AD-58 |                                                                                                                                                                                           |
| SLC7A7    | Upregulated | AD-94 / AD-58 | The solute carrier (SLC) group of membrane transport proteins include 65 families, mostly located on the cell membrane                                                                    |
| SLC15A3   | Upregulated | AD-94 / AD-58 |                                                                                                                                                                                           |
| SLC37A2   | Upregulated | AD-94 / AD-58 |                                                                                                                                                                                           |
| ITGB2     | Upregulated | AD-94 / AD-58 | Integrins are receptors located on the membrane which facilitates cell-extracellular matrix adhesion                                                                                      |
| ITGB7     | Upregulated | AD-94 / AD-58 |                                                                                                                                                                                           |
| OAS1      | Upregulated | MH-25 / AD-58 | 2'-5'-oligoadenylate synthase synthesizes higher oligomers of 2'-5'-oligoadenylates (2-5A) from ATP may play a role in cellular processes such as apoptosis, cell growth, differentiation |
| OAS2      | Upregulated | MH-25 / AD-58 |                                                                                                                                                                                           |
| OAS3      | Upregulated | MH-25 / AD-58 |                                                                                                                                                                                           |

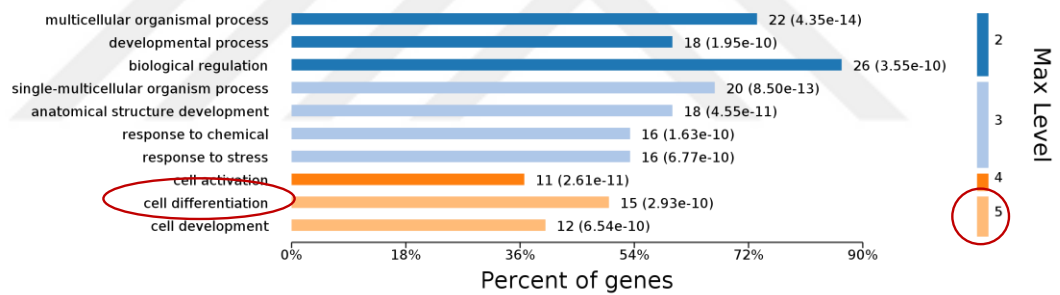
### **3.16 Gene Ontology and Pathway Enrichment Analysis**

To understand the molecular functions, the cellular components, and the determination of biological processes activated upon drug treatments, our differentially expressed gene datasets were further analyzed for gene ontology and pathway enrichment analysis. Correlatively with our previous results, 50% of the differentially expressed genes belonged to cell differentiation processes (**Figure 3.29A, Figure 3.29B**). Enriched proteins of the biological processes revealed that 30% of the gene sets were coding for the immune effective process, which can potentially contribute to an immune response leading by white blood cell differentiation (**Figure 3.29C**). Similarly, enriched molecular function analysis showed that 20% of the genes indicate that myeloid leukocyte activation (**Figure 3.29D**).

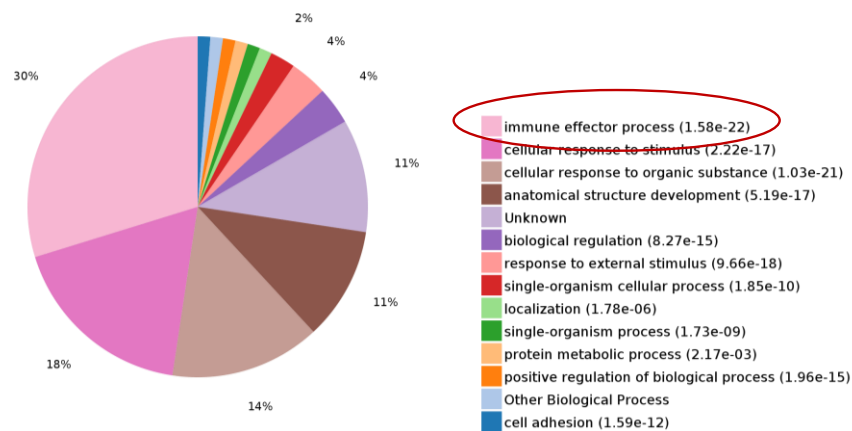
A.



B.



C.



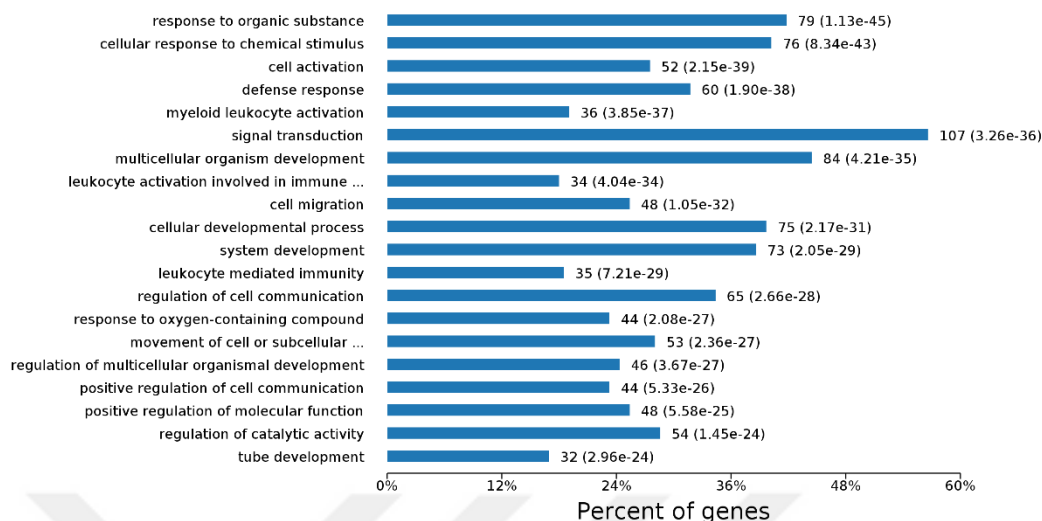


**Figure 3.29:** Gene ontology and pathway enrichment analysis upon LSD1/HDAC6 inhibition.

Enriched gene ontology analysis (**A**), levels of enriched biological process (**B**), proteins of enriched biological processes (**C**), enriched molecular function (**D**). Figures were created using OmicsBean Software.

### 3.17 Validation of RNA-seq Hit Genes by RT-qPCR

To validate the selected hits, we measured the expression levels of several relevant genes by RT-qPCR. The genes were selected based on their relationship with the epigenetic enzyme (LSD1 or HDAC6), their contribution to cell differentiation, or Doxorubicin sensitivity. In accordance, with our RNA-seq analysis, qPCR validation also confirmed the differential expression pattern of the selected genes.

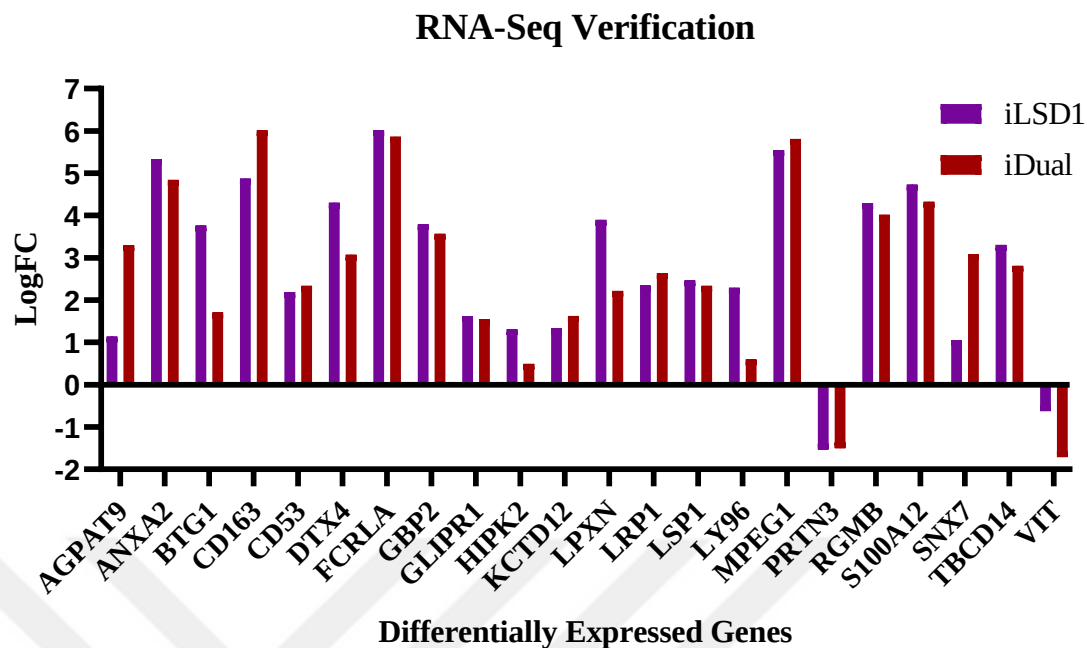


**Figure 3.30:** RT-qPCR analysis of top differentially expressed genes for validation of RNA-Seq hits upon LSD1 inhibition with AD-94 and AD-58 treatment.

Gene expression was normalized to  $\beta$ -actin. Data represented as the average of the mean with duplicates (\* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ , two-way ANOVA).

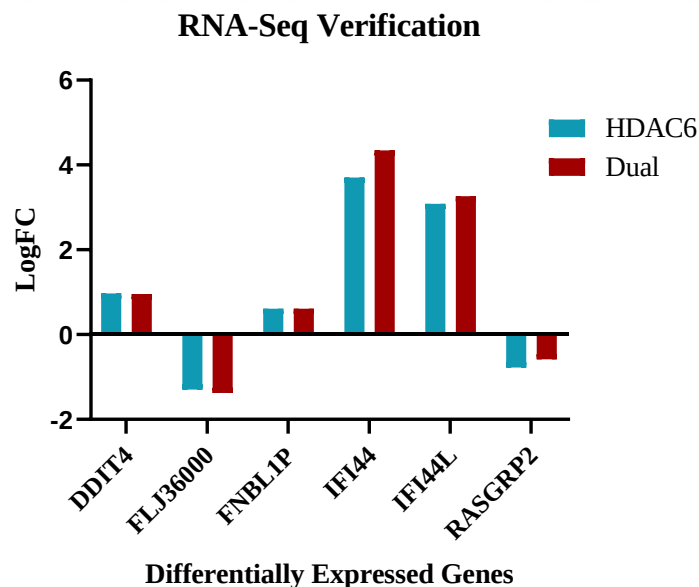


**Figure 3.31:** RT-qPCR analysis of top differentially expressed genes for validation of RNA-Seq hits upon HDAC6 inhibition with MH-25 and AD-58 treatment.

Gene expression was normalized to  $\beta$ -actin. Data represented as the average of the mean with duplicates (\* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ , two-way ANOVA).

qPCR analysis revealed that Doxorubicin sensitizer genes AGPAT9, BTG1, HIPK2, LRP1 were upregulated upon LSD1 inhibition by AD-94 and AD-58 and IFI44L was upregulated upon HDAC6 inhibition (**Figure 3.30, Figure 3.31**). Similarly, monocyte-macrophage differentiation-related gene expressions were significantly increased upon LSD1 inhibition (**Figure 3.30**).



## Chapter 4: DISCUSSION

### 4.1 *LSD1 Inhibition*

Acute myeloid leukemia is a clinically and genetically heterogeneous disease that affecting people all of the ages. AML cases with an annual incidence of 19.000 are expected to continue to increase in the coming years. *De novo* and secondary AML cases are rising due to the increase in the lifespan of the elderly population and the recovery rates of different cancer patients [Miller et al., 2019]. Multiple chromosomal abnormalities and diversity in genetic mutations between AML patients, reduce benefit gained from conventional chemotherapy regimen. Characterizing the heterogeneity of patients is critical for the application of more targeted and individualized therapy.

Chromosome 11, band q23 lesions are among the cytogenetic abnormalities associated with hematopoietic malignancies, and the most prominent gene in this region is the MLL gene. Rearrangements in the MLL gene are associated with pronounced clinical features and poor prognosis [Munoz et al., 2003]. Thus, normal biology and pathology of MLL and MLL fusions are under investigation. The highest fusion rate in acute myeloid leukemia belongs to MLL-AF9 (30%) (**Table 1.1**). As a result of this translocation, MLL protein loses its methyltransferase activity, and its fusion with AF9 is able to recruit AF9 interaction partners to MLL target sites on the DNA. Meanwhile, dysregulation of recruited enzymes may lead to the onset of leukemogenesis.

Among many strategies, manipulating the dependency of AML cells on LSD1 demonstrated a major efficacy so far. Several natural products such as polyphenols, baicalin, resveratrol and geranylgeranoic acid (GGA), and their derivatives were reported to inhibit LSD1 activity *in vitro* [Abdulla et al., 2013; Sakane et al., 2014; Zheng et al., 2016; Duan et al., 2017]. Polyamine analogs showed a strong affinity to bind the FAD-dependent oxidase domain and inhibited LSD1 activity *in vitro* and *in vivo* [Huan et al., 2007]. Metal including inhibitor of rhodium (III) complex 6 was reported to block LSD1 activity and had an anti-proliferative effect in prostate cancer cells, however, bioavailability was a big drawback *in vivo* [Yang et al., 2017]. MAO inactivators Pargyline, Tranylcypromine, and Phenelzine were early identified LSD1 inhibitors. The chemical structure of Tranylcypromine has undergone many changes in order to increase

specification after being the first discovered LSD1 inhibitor. Finally, Tranylcypromine derivatives have been attempted to be designed to avoid side effects via inhibition of MAO [Feng et al., 2016; Ji et al., 2017]. Among all these derivatives, if the cyclopropylamine ring carries substitutions on the same carbon (similar to MH-18), the resulting molecule loses its ability to inhibit LSD1 (**Figure 3.1**), also the cyclopropylamine ring structure that has substitutions on the different carbons should be preserved for irreversible LSD1 inhibition (**Figure 1.9A**, **Figure 1.9B**, **Figure 1.9C**) [Culhane et al., 2010]. On the other hand, SP-2509 does not contain a cyclopropylamine ring and can only inhibit LSD1 reversibly (**Figure 1.9D**).

Several LSD1 inhibitors are synthesized to date and they are shown to successfully inhibit LSD1 activity. For instance, the introduction of sulfonamide groups in the TCP benzene ring was reported to increase LSD1 inhibition potency. Interestingly, despite having achieved a value of 0.12  $\mu\text{M}$  in inhibiting LSD1 enzymatic activity, a9 did not show similar success in inhibiting the growth of AML cells. This result has been associated with the absence of lipophilic groups, thus the same research group reported that the addition of lipophilic benzyl or benzene groups, led synthesis of a10 molecule. The smart design of LSD1 inhibition with a10 achieved a value of 0.029  $\mu\text{M}$  in inhibiting LSD1 enzymatic activity and showed 15.4  $\mu\text{M}$  half inhibitory concentration following induced cell cycle arrest at the G0/G1 phase in AML cells [Liang et al., 2020]. Similarly, our study indicated that the removal of a hydroxyl group from GSK2879552 and the addition of a benzene derivative, imidazole resulted in the formation of AD-94 (**Figure 3.1**). AD-94 improved LSD1 inhibiting potential (**Table 3.1**) and effecting the dose in cell growth compared to GSK2879552 (**Table 3.2**). Therefore, our results revealed that the addition of lipophilic groups facilitates easy entry into the cell and increases efficacy. Similarly, the conversion of imidazole, which results in AD-94 improved LSD1 stabilization when compared with GSK2879552 in AML cells (**Figure 3.4A**).

Further analysis is required to determine the specificity of adding the imidazole group to the GSK2879552 compound against other MAOs. Although specificity testing has not been performed, the expression of MAOs, which are mostly localized in the mitochondria is mostly negligible in THP-1 cells [[Protein Atlas, 2020](#)]. For this reason, the potential expected off-target candidates are eliminated in these cells.

The MLL gene has been found to be highly active and involved in several translocations in AML [Matsuo et al., 1997]. THP-1 was the first established leukemic

cell line with acute monocytic leukemia derived from a 1-year old boy in 1980 [Tsuchiya et al., 1980]. THP-1 cells carry t (9; 11) (p22; q23) translocation and express MLL-AF9 fusion protein. MOLM-13, on the other hand, is another acute monocytic leukemia with (9,11) translocation and derived from a 20-year old male [Matsuo et al., 1997].

THP-1 and MOLM-13 cell lines were chosen due to their infant monocytic characteristics. Thus, both cell lines have been confirmed to be the most convenient cellular models for studying monocytic differentiation and leukemogenesis [Fleischmann et al., 2014; Chanput et al., 2014].

The heterogeneous population in AML consists of incompletely differentiated progenitors that suppress normal hematopoiesis [Kerenyi et al., 2013]. LSD1 was reported to involve in the terminal differentiation of several lineages, through dynamically interacting with growth factor independent (GFI) proteins [Saleque et al., 2007]. LSD1 is required for terminal differentiation in hematopoiesis and dysregulated LSD1 activity is correlated with myeloproliferative disorders [Kozub et al., 2017]. Nevertheless, LSD1 was reported to be highly expressed in leukemia stem cells, thus several studies revealed that LSD1 inhibition is a highly effective method for targeting AML [Goardon et al., 2011]. LSD1 inhibition in AML was reported to enhance differentiation [Schenk et al., 2012]. In accordance, elevated levels of myeloid-differentiation-associated genes were reported following LSD1 inhibition by other groups [Smitheman et al., 2019]. The cumulative data on LSD1 identified two macrophage markers CD86 and CD11b as cellular biomarkers for LSD1 inhibition [Lynch et al., 2013]. Despite its indispensable role in hematopoiesis, LSD1 inhibition in cells with dysregulated activity improved monocyte differentiation through activation of myeloid lineage marker genes (**Figure 3.14A, Figure 3.14B**).

LSD1 has a dual life: it is either a transcriptional repressor through H3K4 demethylation or, an activator through H3K9 demethylation [Magliulo et al., 2018]. Interestingly, cells appear to respond differentially in response to LSD1 inhibition for the methylation of these two substrates most likely as a result of different genetic backgrounds they possess. For instance, while LSD1 inhibition through siRNA has been shown to increase H3K4me1 and H3K4me2 expression in a dose-dependent manner in ALL cells [Zou et al., 2017], and induced global H3K4me2, H3K4me3, and H3K9me2 levels in the AML cell line OCI-AML3 [Fiskus et al., 2014], another study showed that there was no specific accumulation of H3K4me1/H3K4me2 upon LSD1 inhibition with

OG86 (a specific TCP-derivative LSD1 inhibitor) in THP-1 cells [Maiques-Diaz et al., 2018]. In accordance with this study, we found no significant increase in global H3K4me2 levels in THP-1 cells upon LSD1 inhibition (**Figure 3.11**). Yet, higher global H3K4me2 was observed in AD-94/iLSD1 and AD-58/iDual treated MOLM-13 cells (**Figure 3.12**). Comparatively, the TCP derivative a10 molecule induced H3K4me2 and H3K9me2 levels in the MV4-11 cell line whereas, TCP treatment only induced H3K9me2 levels significantly in THP-1 cells (**Figure 3.10**). H3K4 methylation in the promoter region has been associated with transcriptional activation [Soares et al., 2017]. Previous studies have shown an increase in the expression of CD86 and CD11b through H3K4me2 accumulation on the gene promoters following LSD1 inhibition [Fang et al., 2017]. It is yet to be determined whether our inhibitors, AD-94/iLSD1 and ADXX/iDual, also promotes increased H3K4me2 accumulation on these gene promoters.

While epigenetic drugs are usually not cytotoxic on their own, they may prime the cells to a more normal-like state potentiating the chemotherapeutic effects of cytotoxic drugs. The global change of chromatin organization appears to be responsible from the increased efficacy of these therapeutics [Li et al., 2017]. For example, LSD1 inhibition with GSK2879552 and ATRA combination was reported to enhance the anti-tumor activity, and lead caspase-mediated cell death in AML cells. The same study revealed that blast colony-forming abilities were impaired in patient-derived samples treated with GSK2879552 and ATRA [Smitheman et al., 2019]. Similarly, another LSD1 inhibitor INCB059872 was synergistic with ATRA in human AML cell lines and primary AML cells. This combination also reduced tumor growth in the THP-1 xenograft model of AML [Ye et al., 2016]. In addition, combined pharmacological inhibition of LSD1 and mammalian target of rapamycin (mTOR) was reported to impair MLL-rearranged AML growth [Deb et al., 2019]. Therefore, LSD1 inhibition in combination with other molecules strike as an attractive strategy for leukemia.

Fortunately, this strategy is not only limited to leukemia. Several studies have reported synergy between LSD1 inhibitors and cytotoxic agents in solid tumors as well. For instance, suppression of LSD1 enhanced the cytotoxic effects of Regorafenib in liver cancer [Wu et al., 2019]. Furthermore, inhibition of LSD1 reversed Doxorubicin and Paclitaxel resistance in breast cancers and co-treatment of GSK-LSD1 with Doxorubicin or Paclitaxel reduced the growth of tumorspheres [Verigos et al., 2019].

While the combined action of LSD1 inhibitors with other drugs in various cancers have been reported, to our knowledge, the synergistic effect of Doxorubicin and LSD1 inhibition on AML cells was described for the first time with our study at the time of this thesis submission (**Figure 3.16D, Figure 3.19A**). Similarly, the additive cytotoxicity of Paclitaxel and LSD1 inhibitors on AML cells has not been reported previously (**Figure 3.16C**). Since Doxorubicin is the second-line used drug by AML patients receiving chemotherapy with 7-3 protocol, we believe the synergy with LSD1 inhibition is a significant finding with clinical importance.

In order to further analyse how Doxorubicin and LSD1 inhibitors synergize to induce cell death; potential targets were examined to understand the underlying molecular mechanism.

As mentioned earlier, Doxorubicin has three major mechanism in the context of cell death. Doxorubicin is a DNA intercalator, Topoisomerase II inhibitor and a ROS generator. DNA intercalation and Topoisomerase II inhibition usually lead DNA damage.

DNA damage response usually starts with the detection of DSBs and phosphorylation of variant histone H2A.X by ATM kinases [Jackson and Bartek, 2009; Ciccia and Elledge, 2010]. LSD1 was reported to colocalize with the damage site for transcriptional inactivation through H3K4 demethylation. However, LSD1 recruitment is H2A.X-independent and promoted by Ring finger protein (RNF168). LSD1 knockdown also sensitizes cells to DSBs from  $\gamma$ -irradiation and induce homologous recombination [Mosammaparast et al., 2013]. Similarly, our work suggested that inhibition of LSD1 activity, increased the expression of homology-directed repair (**Figure 3.23A**). However, our H2A.X staining indicated that, LSD1 inhibition sensitized THP-1 cells to DSBs upon Doxorubicin administration in an H2A.X-independent manner (**Figure 3.16D, Figure 3.21A**).

Reactive oxygen species may oxidase several biomolecules, leading to cell death [Cappetta et al., 2017]. LSD1 is a FAD-dependent enzyme and its demethylase activity results in the removal of hydrogens ( $2H^+$ ) on the methyl group and binding to FAD. The hydrogens of this FADH<sub>2</sub> molecule are bound with free oxygen (O<sub>2</sub>) in the environment and FAD is released again for the continuity of the reaction. Thus, typically elevated LSD1 activity leads to increased formation of endogenic ROS, such as hydrogen peroxide H<sub>2</sub>O<sub>2</sub>, and LSD1 is a rare oxidase candidate for ROS production in the nucleus [Duquette et al., 2018]. LSD1 is known to accumulate at DNA damage sites [Mosammaparast et al.,

2013] and release ROS. LSD1 inhibition was reported to delay ROS generation upon DSBs [Duquette et al., 2018]. Pharmacological inhibition of LSD1 and mTOR was reported to prevent hemolysis in sickle cell disease via mitochondria retention and reduced ROS levels [Jagadeeswaran et al., 2017]. Our study indicated that LSD1 inhibition reduced ROS generation upon DNA damage by Doxorubicin administration, however, LSD1 inhibition itself did not affect ROS levels (**Figure 3.22A**). H<sub>2</sub>O<sub>2</sub> release triggered by LSD1, modulates sensor proteins such as Ku80 or Nbs1 that will initiate the repair path [Duquette et al., 2018]. However, it remains to be determined how reduced H<sub>2</sub>O<sub>2</sub> disrupts the DNA repair mechanism in AML cells.

AD-94/iLSD1 treatment induces many unique transcriptional changes, including the upregulation of genes involved in cellular differentiation. Although the exact cell pathways induced by AD-94 are not yet fully characterized, they include macrophage differentiation, based on our expression profiling. Our OmicsBean gene ontology analysis revealed that nearly 50% of the DEGs were associated with the cell differentiation process (**Figure 3.29**). Enriched KEGG Pathway analysis indicated that the expression of AML-driver genes decreased after LSD1 inhibition. Based on these results, LSD1 inhibition alone, induces macrophage differentiation, while at the same time pre-sensitizing cells for Doxorubicin. Several Doxorubicin sensitizer genes were upregulated upon LSD1 inhibition; however, it remains to be unknown whether these gene expressions are directly regulated by LSD1 or not. These sensitizer genes include BTG1, whose knock-down was recently shown to induce leukemia development and BTG-family overexpression was reported to be responsible for Doxorubicin-driven cell death [Moorman et al., 2012; Yuniati et al., 2019].

Another gene AGPAT9 is an acyl glycerol transferase, which is mainly expressed in the adipose tissue. AGPAT9 was reported to be overexpressed in poorly invasive MCF-7 cells, however, its expression was decreased upon doxorubicin resistance. Stable expression of the AGPAT9 gene in the Doxorubicin resistant MCF-7 cells significantly reduced the IC<sub>50</sub> value for Doxorubicin [Fan et al., 2015]. Therefore, the increase in the AGPAT9 gene expression following inhibitor therapy may be responsible for the increased sensitivity of Doxorubicin on leukemia cells.

Homeodomain-interacting protein kinase-2 (HIPK2) belongs to a conserved family of serine/threonine kinases and is considered to be a tumor suppressor and actively takes part in apoptosis, proliferation, differentiation, and metastasis. HIPK2 knockdown was

reported to induce resistance to multiple anticancer agents, including Doxorubicin and Cisplatin in esophageal squamous cell carcinoma [Zhang et al., 2018]. In accordance, HIPK2 overexpression was reported to our inhibitors and reduce cell viability following Cisplatin treatment. Therefore HIPK2 regulation via LSD1 may be a mechanism for the synergy observed between Doxorubicin on AML cells.

Proto-oncogene tyrosine-protein kinase is a gene, which encodes for a ROS1 receptor that controls cell growth and differentiation. ROS1 knockdown was reported to sensitize breast cancer cells against Doxorubicin [Tiash et al., 2016] and similarly, this gene was downregulated in our AD-94 treated cells.

Low-density lipoprotein receptor-related protein 1 (LRP1), is an endocytic receptor is involved in including lipid homeostasis, and clearance of dead cells. A recent study revealed that increased efflux transporter and upregulation of the LRP1 gene leads to higher accumulation of Doxorubicin and increases drug's efficacy in lung adenocarcinoma. As mentioned in Table 3.6, LRP1 expression was upregulated upon LSD1 inhibition and may contribute to Doxorubicin accumulation in AML cells.

Among these Doxorubicin sensitizers, a notable gene, The arachidonate 5-lipoxygenase gene (ALOX5), was upregulated in response to LSD1 inhibition. ALOX5 is a ROS generating non-heme iron-containing enzyme, which is highly down-regulated on MLL-rearranged leukemias. Its overexpression not only reduces MLL-AF9 colony-forming capacity but also exhibits an anti-tumor effect., , Furthermore, elevated levels of ALOX5 sensitizes AML cells for both Cytarabine and Doxorubicin (7-3 protocol in AML chemotherapy regimen) [Wang et al., 2017]. In accordance, ALOX5 was upregulated in AD-94 treated cells, which might be considered as the key gene involved in the sensitization of Doxorubicin.

While a single change in expression of any of the aforementioned genes might be responsible for the sensitization of AML cells to Doxorubicin, the cumulative effect of their expression changes or a particular combination may also contribute to the observed synergy. Therefore, it is yet to be determined which one of these scenarios is correct. Indeed, among all genes ALOX5 appears to stand out given its importance based on previous literature.

Overall, while we still need to investigate how the synergy occurs, our findings strongly hint towards the usage of LSD1 inhibitors in combination with Doxorubicin as a rational strategy for AML treatment.

## 4.2 HDAC6 Inhibition

HDACs are an important group of erasers involved in many biological processes. Their activity controls proliferation, differentiation, and cell death pathways including apoptosis. Compared to other anticancer agents, HDAC inhibitors have a wider effect on individual pathways on different tumor types. Therefore, many heterogeneities in cancer cells lead to higher sensitivity against HDAC inhibition more than healthy cells [Xu et al., 2007].

To date, few HDAC inhibitors such as Ricolinostat, Entinostat, or Romidepsin have been found to be specific to a particular class of HDACs. Hydroxamic acids, benzamides, cyclic tetrapeptides, or short-chain fatty-acids usually target class I, II, or IV HDACs due to their potential Zinc<sup>+</sup> chelating ability. Hydroxamic acid-containing HDAC inhibitors mostly achieved clinical trials while 3 of them (SAHA, Belinostat, Panobinostat) were approved for the treatment of leukemia [Eckschlager et al., 2017].

In our study, conversion of the carboxylic acid to a hydroxamic acid in GSK2879552 generated a novel molecule MH-25 for HDAC inhibition (**Figure 3.2**). The inhibitor has been shown to exhibit a great affinity for class II HDACs (HDAC6) compared to class I HDACs (HDAC1, 3, and 8) in with or without cell-based assays (**Table 3.1, Figure 3.4**). MH-25 shown to be stabilized HDAC6 enzyme in THP-1 cells similarly with Ricolinostat. Despite that Ricolinostat has a high affinity for HDAC6 in the extracellular environment, it has been shown to stabilized class I HDACs in the intracellular environment (**Figure 3.4**). The lower IC<sub>50</sub> value of Ricolinostat also suggested that the drug has higher toxicity due to its off-targets within the cell (**Table 3.2**).

HDAC inhibitors have been reported to profoundly reduce cell proliferation on leukemic cells [Sakajiri et al., 2005]. Their inhibition generally reduces the expression of EMT related genes and leads apoptosis/necroptosis [Natarajan et al., 2018]. Despite the effective preclinical results for HDAC inhibitors, unfortunately their monotherapy seems to be limited in AML. Therefore, combination strategies with several anticancer drugs showing significant anti-leukemic activity, are being tested in clinical trials [San Jose-Eneriz et al., 2019]. For instance, the FDA approved pan-HDAC inhibitor, SAHA combination with Idarubicin successfully completed phase I study in relapsed leukemia [Kadia et al., 2010]. Furthermore, Cytarabine's addition to this combination passed phase

II study in patients with newly diagnosed acute myelogenous leukemia in 2012 [Garcia-Manero et al., 2012].

Another pan-HDAC inhibitor Panobinostat combination with Cytarabine and Mitoxantrone was attempted in AML patients with two clinical trials [Schlenk et al., 2018]. 7-3 protocol with Cytarabine, Daunorubicin, and Panobinostat treatment in older patients with AML was reported to be well-tolerated, as a phase I study result [Wieduwilt et al., 2019]. Similarly, Cytarabine, Idarubicin, and Panobinostat combination passed phase II studies for elderly AML patients [Ocio et al., 2015]. However, our study indicated that co-treatments with HDAC6 inhibition and Cytarabine, Paclitaxel, Cisplatin, or Etoposide did not result in synergy in AML cells (**Figure 3.16A, B, C, Figure 3.20B**) suggesting that triple treatment methodologies may be necessary. Not surprisingly, this strategy is being tested and clinical trials are on their way using DNMT inhibitor Azacitidine, LSD1 inhibitor GSK2879552 in combination with Panobinostat for the treatment of AML [Garcia-Manero et al., 2017; Bewersdorf et al., 2019].

To date, while HDAC inhibitors are not yet tested in combination with Doxorubicin in the clinic, there are a number of preclinical studies with promising results. These studies have shown that Panobinostat and Doxorubicin synergize and induce cell death via dysregulation of DNA repair in AML [Maiso et al., 2009]. Class I and II HDACs perform deacetylation upon DNA DSBs, and at the break sites, the absence of HDACs was reported to disrupt DSB repair [Bhaskara, 2015]. Furthermore, a selective HDAC6 inhibitor, MPT0G211, was shown to sensitize HL-60 cells to Doxorubicin induced-cell death. Interestingly, MPT0G211 impaired DNA repair machinery initiated by Doxorubicin administration via disruption of Ku70-Bax protein binding and co-treatment suppressed tumor growth both *in vitro* and *in vivo* [Tu et al., 2018]. Correlatively, our results demonstrated that although HDAC6 inhibition itself had no effect on H2AX activation, it slightly potentiated Doxorubicin-induced double-strand breaks (**Figure 3.21B**).

Cells activate cell cycle checkpoints against genotoxic agents. Checkpoints, which allow DNA to be repaired before replication or mitosis, provide short-term pauses, and lead activation of several tumor-suppressor genes including p53 [Kastan et al., 1992]. Normally, p53 is inactivated by its negative regulator MDM2, and upon stress, it activates downstream regulators p21 and GADD45 [el-Deiry et al., 1993; Zhan et al., 1994]. p21 is known to have roles in transcriptional regulation following DNA damage [Karimian et

al., 1996]. GADD45 protein acts as a sensor and modulates DNA repair, cell cycle, or apoptosis [Jin et al., 2003]. Our study indicated that HDAC6 inhibition with MH-25 resulted in hyperacetylation of  $\alpha$ -tubulins, therefore stabilization of microtubules (**Figure 3.13**). We further observed that this stabilization leads to higher expression of p21 (**Figure 3.15**) and GADD45A (**Figure 3.23B**), suggesting that HDAC6 inhibition activates stressful growth arrest conditions. On the other hand, MH-25 induced expression of several HDAC6-regulated genes including RASGRP, DDIT4, FNBP1L, and IFI44 [Espino et al., 2005; Namdar et al., 2010; Lin et al., 2016; Klionsky et al., 2011]. Indeed, HDAC6 inhibition changed over a smaller number of genes compared to LSD1 inhibition, supporting that MH-25 generally targets a cytoplasmic HDAC rather than nucleic HDAC, correlatively exhibits fewer changes on the transcriptome.

RNA-seq results revealed that HDAC6 inhibition led activation of IFI44L, which is a novel tumor suppressor, affecting cancer stem cell characteristics and Doxorubicin sensitivity. Overexpression of IFI44L increases chemosensitivity against Doxorubicin in hepatocellular carcinoma, supporting that a similar sensitization might explain the additive effect of Doxorubicin and HDAC6 inhibition on AML cells [Huang et al., 2018]. While there was no  $\gamma$ HA2X activation on HDAC6 inhibition alone, there was a significant increase in the expression of genes associated with DNA damage including DDIT4. DDIT4 is expressed under several molecular stress conditions such as exposure to DNA damaging agents Etoposide [Wang et al., 2003]. As discussed in chapter 4.1, an important chemotherapy sensitizer ALOX5, also appeared as an upregulated gene in HDAC6 treated cells. These results indicate that HDAC6 inhibition leads programmed cell death in AML cells by stabilization of tubulins, disrupting the DNA repair mechanism, and sensitizing against Doxorubicin via the upregulation of ALOX5, DDIT4, and IFI44L.

Overall, our study highlights the role and importance of HDAC6 inhibition combined with Doxorubicin as a novel therapeutic modality in AML patients.

### **4.3 *iDual Inhibition***

Studies to date have clearly shown that most diseases are regulated by interconnected pathways and their treatment modalities can be improved by the use of multiple drugs targeting several different candidates in these networks. Therefore, there is a growing interest in designing dual inhibitors that hit two birds with one stone. Dual targeting is

usually advantageous because drugs have increased efficacies, less side effects compared to drug combination and reduced acquisition of drug resistance, yet it also comes with a cost and there are challenges such as balancing biological activities on the desired proteins. Previously, several novel dual-targeting inhibitors were reported to show anti-leukemic activity. Dual inhibition of PI3K and mTORC1/2 signaling by NVP-BEZ235 was reported to be considered as a new therapeutic strategy for AML [Chapuis et al., 2010]. Similarly, a dual inhibitor of Class IA phosphatidylinositol 3-kinase and mTOR (PI-103) demonstrated antileukemic activity in AML [Park et al., 2008]. AS602868, a dual inhibitor of IKK2 and FLT3 played a crucial role in blocking the pathogenesis of AML and T-cell leukemia [Griessinger et al., 2007; Frelin et al., 2003].

As mentioned earlier, epigenetic drugs may prime cancer cells to well-known chemotherapies. Epigenetic regulations have been broadly identified for characterizing abnormal growth in hematologic malignancies, thus inhibition of epigenetic modulators drew attention for the treatment of leukemia [Fong et al., 2014]. There are several investigations on dual inhibition in leukemia, however targeting of two different enzymes with different roles, is remained limited.

Dual bromodomain/HDAC inhibition demonstrated antiproliferative effects against two human leukemia cell line K-562 and MV4-11 [Zhang et al., 2016]. Reversible euchromatic histone-lysine N-methyltransferase 2 (EHMT2), also known as G9a and DNMT dual inhibitor CM-272 inhibited AML cell proliferation and induced apoptosis. CM-272 was also reported to exert a potent *in vivo* activity and increase the survival rate *in vivo* [San Jose-Eneriz et al., 2017]. Another study revealed the synthesis of Tranylcypromine derivatives as novel LSD1/HDAC dual inhibitor for cancer treatment [Duan et al., 2017]. Similarly, our study indicated synthesis and characterization of GSK2879552 derivatives as novel and clinically useful LSD1/HDAC6 dual inhibitor for AML therapy.

Removal of carboxylic acid and following the addition of hydroxamic acid in GSK2879552 resulted in the generation of AD-58, a dual LSD1/HDAC6 inhibitor (**Figure 3.3**). Based on the  $IC_{50}$  values, the conversion of hydroxamic acid in GSK2879552 molecule improved LSD1 binding potential, in addition, this conversion provided a binding ability to HDAC6 (**Table 3.1**). Dual inhibitor led to the stabilization of both LSD1 and HDAC6 in AML cells (**Figure 3.4**). Our data demonstrated that AD-58 exposure resulted in dual inhibition, stimulating histone methylation and tubulin

acetylation. As mentioned earlier, LSD1 sustains oncogenic potential of leukemia stem cells [Fang et al., 2017], consistent with these results dual inhibition through AD-58 induced macrophage differentiation in AML cells (**Figure 3.14**). Several studies revealed that AML cells have a high dependency on LSD1 and HDACs [Feng et al., 2016; Kruger et al., 2013; Hackanson et al., 2012; San Jose-Eneriz, et al., 2019], and parallel to these previous findings, targeting LSD1 and HDAC6 dramatically attenuated cell proliferation on AML in our studies (**Figure 3.8**). Furthermore, other leukemic cell lines such as CML and T-cell leukemia were also observed to be sensitive against this dual inhibition (**Figure 3.9**)

As mentioned in chapter 4.1 and 4.2, targeting LSD1 or HDAC6 in combination with several cytotoxic agents was investigated, and among the drugs that were tested Doxorubicin was the only one significantly increasing anti-leukemic activity (**Figure 3.16D**). Dual inhibition and Doxorubicin combination also induced Caspase 3-7 activity, suggesting that LSD1 and HDAC6 inhibition further sensitized AML cells for Doxorubicin exposure and led apoptosis (**Figure 3.17, Figure 3.18**).

LSD1 is known to be colocalized with the damage site upon double-strand breaks for transcriptional inactivation and this recruitment was reported to occur as H2A.X-independently [Mosammaparast et al., 2013]. Class I and II HDACs perform deacetylation upon DNA DSBs for transcriptional inactivation, immediately at break sites, and this was reported to be crucial for DSB repair [Bhaskara, 2015]. Given that all, dual inhibition of LSD1 and HDAC6 sensitized AML cells to Doxorubicin  $\gamma$ H2AX-independently, suggesting a deeper molecular investigation is required.

HDAC inhibition has been previously reported to increase ROS generation [Ping Dou, 2014]. On the other hand, LSD1 inhibition decreased Doxorubicin-induced ROS levels (**Figure 3.22A**). Both enzyme inhibition through AD-58 and their combination with Doxorubicin revealed no significant change in the ROS generation in AML cells (**Figure 3.22C**).

DNA damage screen revealed that dual enzyme inhibition induced homology-directed repair through RAD51 activation (**Figure 3.23D**). Multiple genes, which differentially expressed in iLSD1 and iHDAC6 treated cells also appeared in iDual treatments, supporting that the inhibitors have common targets within the cell. As mentioned in chapter 4.1, known to be Doxorubicin sensitizer genes ALOX5, AGPAT-9, BTG-1,

HIPK2, LRP-1, ROS-1 (similarly in AD-94/iLSD1) and DDIT4, IFI44 (similarly in MH-25/iHDAC6) were differentially expressed in the same direction in iDual treated cells. Therefore, the iDual group, with the highest number of sensitivity-related genes, resulted in a higher synergy with Doxorubicin (**Figure 3.16D**, **Figure 3.28**).

Taken together, our current results highlight the role of LSD1 and HDAC6 inhibition in AML and strongly support the investigation of potential Doxorubicin co-treatment with our novel inhibitors for AML patients. Our co-treatment modalities seem to disrupt leukemogenesis via different mechanisms and act synergistically in combination.



## Chapter 5:

### CONCLUSIONS

Epigenetic modifiers broadly influence dysregulated AML transcriptome. In this category, several inhibitors compose promising treatment modalities for AML. Herein, we develop novel small molecules regulating LSD1, HDAC6, and their dual inhibition and characterize their biological functions in AML cells. Obtained data indicated that pharmacological inhibition of LSD1 and HDAC6 reduces leukemogenic activity. Based on the combination results, both inhibitions synergistically potentiate AML cells for clinically used Doxorubicin. Epigenetic inhibitors are at the beginning of clinical development; however, our findings with the underlying mechanism of actions may provide future applications for leukemia therapy.

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