Identification and development of small molecule therapies for the treatment of acute myeloid leukaemia

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Scientific environment

During my PhD I was affiliated with the Signalling-Targeted therapy group, headed by Prof. Bjørn Tore Gjertsen, located at the Department of Clinical Science, University of Bergen. This group is part of the Centre for Cancer Biomarkers (CCBIO) at the University of Bergen. My position was funded by a PhD-grant from the University of Bergen. My main supervisor was Prof. Bjørn-Tore Gjertsen and co-supervisor was Dr. Vibeke Andresen.

Much of the work during my PhD was performed in close collaboration with the Preclinical Oncology Systems group headed by Prof. Emmet McCormack, at the Department of Clinical Science, University of Bergen.

The metallodrug project was performed in collaboration the Bjørsvik Research Group headed by Prof. Hans-René Bjørsvik, at the Department of Chemistry, University of Bergen.

The majority of the thesis was written while I was employed and supported by KinN Therapeutics AS.

Centre for
Cancer Biomarkers
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Contents

Abbreviations

AML – Acute Myeloid Leukaemia

APL – Acute Promyelocytic Leukaemia

Ara-C – Cytarabine

BNML – Brown Norway myelocytic leukemia

CDX – Cell line derived xenograft

CML – Chronic Myeloid Leukaemia

CR – Complete remission

DABA – Dimethylbenz[a]anthracene

DSB – Double strand breaks

DSS – Drug sensitivity score

DSRT – Drug sensitivity and resistance testing

HAT – histone acetyl transferase

HDAC – histone deacytelase

HDACi – histone deacytelase inhibitor

HDM2 – human double minute 2

HR – homologous recombination

HU – Hydroxyurea

IC – intensive chemotherapy

ITD – internal tandem duplicate

LSC – leukemic stem cells

MRD – minimal residual disease

NHC – N-heterocylic carbene

NPM1 – nucleophosmin 1

NSG – NOD/SCID IL2rγ^{null} (NSG)

PDX – patient derived xenograft

PS – phosphatidylserine

RARA – retinoic acid receptor alpha

RPA – replication protein A

R/R-AML – relapse or refractory AML

SDM – serdemetan

t-AML – therapy related AML

TKI – tyrosine kinase inhibitor

VPA – valproic acid

Abstract

Acute myeloid leukaemia (AML) is a cancer of the bone marrow in which immature myeloid cells exhibit uncontrolled proliferation and failure to differentiate. The global 5-year overall survival rate for AML ranges between 25 – 35% indicating the urgent need for novel therapeutic alternatives. Current therapies include bone marrow transplant (for a minority of suitable patients), chemotherapy and a handful of small molecule drugs approved for selected patient subgroups. Poor survival rates and limited therapy response are partially attributed to inter- and intra- patient heterogeneity. The concept of personalised medicine has emerged as one potential strategy to overcome the diversity of AML patients. For this approach to succeed novel small molecules with unique anti-leukemic properties must be developed to expand the arsenal of clinical alternatives.

The primary objective of the thesis was to identify and develop small molecule therapies for the treatment of AML.

In paper I we supported the development of novel small molecules and investigated their cytotoxic properties in AML cell lines. N-heterocyclic carbenes were generated and complexed with silver using a novel multi-step synthetic pathway to produce the putative metallodrugs, NHC-1 and NHC-2. Dose response curves were generated for each of the compounds to determine the IC_{50} of each compound in the AML cell lines, MOLM-13 and HL-60. Cell death induction was characterised as rapid and associated with apoptotic nuclear morphology. Interestingly, we also observed increased phosphatidylserine expression in HL-60 cells treated with the silver NHC complexes as compared with cytarabine.

In paper II we identify the tryptamine derivative, Serdemetan (SDM), as a candidate for small molecule therapy in AML. SDM was originally discovered as part of an internal drug development program at Janssen Pharmaceutical, attempting to identify novel Hdm2 inhibitors for use in solid cancers. In AML cell lines and patient samples SDM activity varied independent of p53 status, suggesting the agent may have additional molecular targets. SDM was well tolerated and significantly prolonged

survival in preclinical models of AML including the syngeneic BNML rat model and MOLM-13 xenograft, further indicating the agent's suitability for development in AML. Interestingly, our studies revealed novel mechanisms of action including upregulation of autophagy markers and depletion of Akt1 expression.

In paper III we propose repositioning Hydroxyurea (HU) and Valproic Acid (VPA) as a combination therapy for the treatment of AML. Both agents have previously been trailed clinically in AML and are generally well tolerated. HU and VPA combined to induce synergistic cell death in multiple AML cell lines. Unlike SDM, combination induced apoptosis and proliferation arrest appeared partially dependent on wildtype p53 expression. Mechanistic studies revealed that VPA dramatically enhances HU induced DNA double strand breaks, likely by downregulating the homologous recombination protein, Rad51. The synergistic efficacy of the combination appeared to be maintained *in vivo* as combination therapy was superior to monotherapies in OCI-AML3 and patient derived xenograft models of AML.

Through different selection strategies the work performed in this thesis effectively identified a diverse group of small molecules worthy of further investigation for the treatment of AML. Mechanistic studies provided novel insights into how each of the agents exert their anti-leukemic properties and should guide biomarker development and identification of sensitive patient subgroups. The preclinical animal studies performed in papers II and III provide an important step towards clinical translation.

List of papers

Paper I:

Alexander H. Sandtorv, Calum Leitch, Siv Lise Bedringaas, Bjørn Tore Gjertsen, Hans-René Bjørsvik: **4-Alkylated Silver-N-heterocyclic Carbene (NHC) Complexes with Cytotoxic Effects in Leukemia Cells.** ChemMedChem. 2015 Sep;10(9):1522-7.

Paper II

Calum Leitch, Ingvild Haaland, Mihaela Popa, Pascal Gelebart, Silje Kristiansen, Vibeke Andresen, Øystein Bruserud, Bjørn Tore Gjertsen and Emmet McCormack: **The tryptamine derivative serdemetan induces autophagy, modulates AKT1 and is active in preclinical models of acute myeloid leukaemia.** (Manuscript)

Paper III

Calum Leitch, Tereza Osdal, Vibeke Andresen, Maren Molland, Silje Kristiansen, Xuan Nhi Nguyen, Øystein Bruserud, Bjørn Tore Gjertsen, and Emmet McCormack: **Hydroxyurea synergizes with valproic acid in wild-type p53 acute myeloid leukaemia** *Oncotarget*. 2016 Feb 16; 7(7): 8105–8118.

1. Introduction

1.1 Cancer

Cancer refers to a collection of diseases that share a common origin: the uncontrolled division of autologous cells (1). Mutations and/or chromosomal aberrations lead to changes in cellular physiology that confer a selective advantage in cancer cells over their untransformed counterparts in local tissue (1). Uncontrolled proliferation and resistance to conventional cell death programs is reinforced by the expression and secretion of cytokines or stimulatory factors, resulting in a positive stimulatory feedback loop amongst malignant cells (2, 3). Unmanageable expansion of cancer cells or metastasis and compromise of critical organs or tissues results in mortality.

In principle, cancer may arise within any tissue in the body that contains reproducing cells. This partially accounts for the enormous heterogeneity observed among diseases collectively referred to as cancer. Within each type of cancer, both the particular mutations and the sequence in which they arise can further contribute to heterogeneity (4). The heterogeneity of cancer patients manifest as variable prognosis and therapy response (4).

This thesis investigates and develops strategies for improving small-molecule therapeutic alternatives in the aggressive blood cancer, acute myeloid leukaemia (AML).

1.2 AML

Acute myeloid leukaemia (AML) refers to a form of cancer arising from immature myeloid cells produced in the bone marrow. Figure 1a provides a schematic overview of haematopoiesis, defining and placing myeloid cells in context among other blood cells. Acquisition of genetic insults in myeloid cells blocks differentiation resulting in accumulation and uncontrolled proliferation of leukemic cells (5). A greater understanding of leukemogenesis has revealed that founder mutations arise in seemingly healthy haematopoietic stem cells (HSCs) that mature into genetically

distinct clones all of which may be present at clinical presentation (Figure 1b + c) (6, 7). Accumulated leukemic cells mitigate normal bone marrow function and typically infiltrate circulation (8). Clinical indications of AML can largely be attributed to bone marrow failure with symptoms arising as a consequence of compromised haematopoiesis and associated cytopenia (9). Symptoms may include fatigue, shortness of breath, paleness, and increased propensity for infection (9). The aetiology of AML remains unclear. Radiation exposure, environmental toxins and chemotherapy increase AML incidence in relevant subgroups but these triggers are not present in the greater population (10-12).

AML is a rare but devastating disease. In Europe approximately 4 individuals per 100,000 are diagnosed with the condition each year (13). The median age at diagnosis is 71 years and incidence is slightly higher in males than females (8, 14, 15). Tragically, overall survival rates for AML patients are dismal and correlate strongly with age as illustrated in Table 1 (16). Concerningly in the absence of significant therapeutic breakthroughs the global incidence of AML is expected to double by 2040 (17).

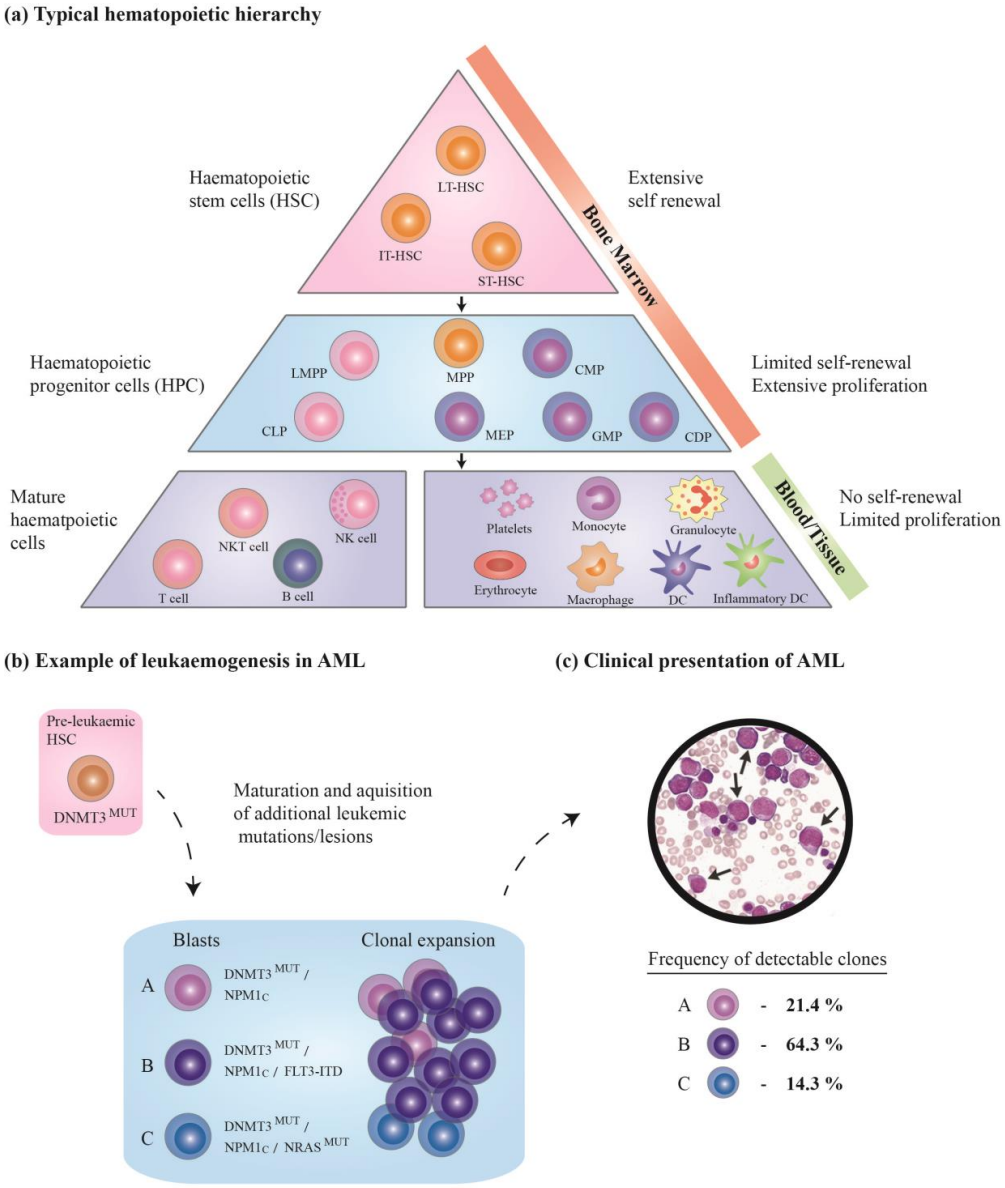

Figure 1. Schematic representation of the haematopoietic hierarchy and depiction of leukaemogenesis and clinical presentation in AML. (a) A schematic overview of the various cell types constituting the hematopoietic hierarchy, indicating tissue location and grouping cells by maturity, capacity for self-renewal and proliferation. LT, long term; IT, intermediate term; ST, short term; HSC, haematopoietic stem cell; MPP, multipotent

progenitors; LMPP, lymphoid-primed multipotent progenitors; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte-erythrocyte progenitor; GMP, granulocyte-monocyte progenitor; CDP, common dendritic cell progenitor; NK, natural killer; DC, Dendritic cell. **(b)** A proposed model of leukaemogenesis where pre-leukaemic HSCs harbouring founder mutations mature and acquire further mutations/lesions leading to transformation and disease manifestation. **(c)** Upon clinical presentation genetic analysis of leukaemia cells may reveal multiple clones carrying unique mutational signatures together contributing to the disease burden at varying frequencies.

Table 1. 5-year overall survival rates for AML patients in Sweden during the periods 1997 – 2006 and 2007 - 2014.

Age Group	Diagnosis Period	5-years overall survival
50 – 59	1997 – 2006	41.9 % (32.7 – 45.5 %)
	2007 - 2014	47.6 % (43.6 – 51.8 %)
60 – 69	1997 – 2006	16.6 % (14 – 19.7 %)
	2007 - 2014	24 % (20.7 – 28 %)
70 – 79	1997 – 2006	3.9 % (2.9 – 5.3 %)
	2007 - 2014	5.5 % (3.9 – 7.7 %)
80+	1997 – 2006	0.8 % (0.3 – 1.7 %)
	2007 - 2014	0.5 % (0.1 – 1.8 %)

Table is adapted from (16). Figures in parenthesis indicate 95% confidence intervals.

1.2.1 Therapeutic Strategies in AML

Novel strategies for cancer treatment continually emerge but the three broad approaches that dominate clinical practice are surgery, radiation therapy and drug-based strategies. Being a cancer of the bone marrow and blood, surgical procedures for treatment of AML are restricted to rare occasions of metastasis and solid tumour

formation (18). Radiation therapy plays an important role for palliative therapy of the AML related myeloid sarcoma and may be used as a part of pre-conditioning AML patients deemed suitable for bone marrow transplant but is of limited utility as a standalone therapy. For these reasons drug-based therapies are of special importance for therapy development in AML. Drug therapy may refer to small molecules, hormones, antibodies, or traditional chemotherapies. The frontline chemotherapy regime for AML patients is composed of two distinct phases. The induction phase comprises 7 days of continuous infusion of the antimetabolite cytarabine (Ara-C), in tandem with 3 days of bolus infusion of an anthracycline, typically daunorubicin (generally referred to as "7 + 3") (5). Complete remission (CR) is defined as less than 5% blasts in the bone marrow and the recovery of normal peripheral blood counts. If CR is achieved, treatment progresses to the consolidation phase comprising high dose Ara-C infusion to eradicate residual disease (9).

The "7 + 3" regime had remained unchanged for decades as the primary care for all AML patients deemed fit enough to tolerate the treatment. Elderly or unfit patients could be offered a milder chemotherapy regime, experimental therapies or palliative care (19). However, recently a wave of new therapies has emerged with as many as 8 new FDA approvals for AML treatment granted since 2017 (20). New approvals have enabled stratification of patients based on mutation status of particular genes but also their fitness and stage in disease development (21, 22). Figure 2 summarises novel therapies recently made available for the treatment of specific subgroups of AML patients. In fit patients, most of the targeted therapies are currently established as combinations or in development in combination with "7 + 3".

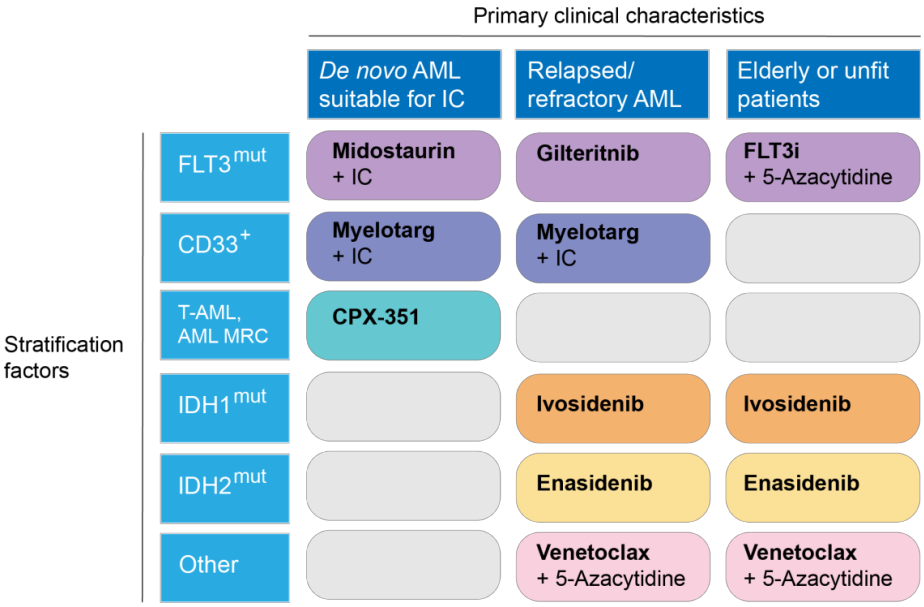

Figure 2. Schematic representation of the recently approved therapeutic alternative for AML patients stratified by molecular and clinical characteristics. IC; intensive chemotherapy. The repertoire of approved clinical agents available for AML patients. Assessment of clinical characteristics and molecular stratification factors should be evaluated at diagnosis but also throughout disease progression to enable a flexible and dynamic approach to disease management and treatment schedules. Midostaurin (Rydapt, FDA approval April 2017), is a multitargeted protein kinase inhibitor to be combined with chemotherapy in patients carrying FLT-3 mutations. Gilteritnib (Xospata, FDA approval November 2018) is a tyrosine kinase inhibitor with high specificity for FLT-3 for relapsed or refractory patients carrying FLT-3 mutation. Gemtuzumab ozogamicin, (Myelotarg, FDA approval September 2017) is a monoclonal antibody-drug conjugate targeting the cell surface marker CD33. CPX-351 (Vyxeos, FDA approval August 2017) is a liposomal formulation of daunorubicin and cytarabine in a fixed concentration. Ivosidenib, also known as (Tibsovo, FDA approval July 2018), is a small molecule inhibitor of isocitrate dehydrogenase-1, IDH1. Enasidenib (AG-221, FDA approval August 2017), is a small molecule inhibitor of isocitrate dehydrogenase-2, IDH2. Venetoclax, (Venclexta or Venclyxto, FDA approval November 2018) is a small molecule inhibitor of the Bcl-2 protein. Figure adapted from (20).

The hedgehog pathway inhibitor, glasdegib, (absent from Figure 2) also received FDA approval in 2018 for treatment of elderly or unfit patients in combination with low dose chemotherapy, though venetoclax plus hypomethylating agents seems more efficient in this patient subgroup and will likely be favoured going forward (23). Whilst the full impact of these new therapies will have to be evaluated over longer periods of use, initial clinical trials indicate significant but modest improvement in relevant patient subgroups (24). Furthermore, several of these agents are only approved by FDA. Actual use of these new agents varies in different countries and is dependent on the reimbursement from heath care insurance providers. As such, an urgent demand remains to bolster and improve the therapeutic repertoire available to clinicians.

1.3 Molecular and cellular features of AML

1.3.1 Genomic Landscape

Cancer is generally associated with changes in the DNA of malignant cells. The causal relationship between genetic abnormalities and the cancer phenotype of single cells is now understood to be more fluid than initially conceived (25, 26). Nevertheless, a detailed description and understanding of the genetic and molecular features of AML is valuable and has greatly informed drug development. DNA sequencing studies have revealed that, on average, de novo AML patients present with 10-15 gene mutations deemed significant for leukaemogenesis and disease maintenance (27, 28). The particular genes involved, their frequency and associated mutations, are variable between and within individual patients. In a sampling of 1540 patients over 5000 driver mutations were identified across 76 genes or specified regions of DNA (29). Genes considered to be recurring and significant can be broadly categorized into 9 subgroups; DNA methylation, spliceosome-complex genes, tumour suppressors, cohesion-complex genes, signalling pathways, chromatin modification, myeloid transcription factors, transcription factor fusions and mutations of the nucleophosmin (NPM1) gene (27). See Figure 3.

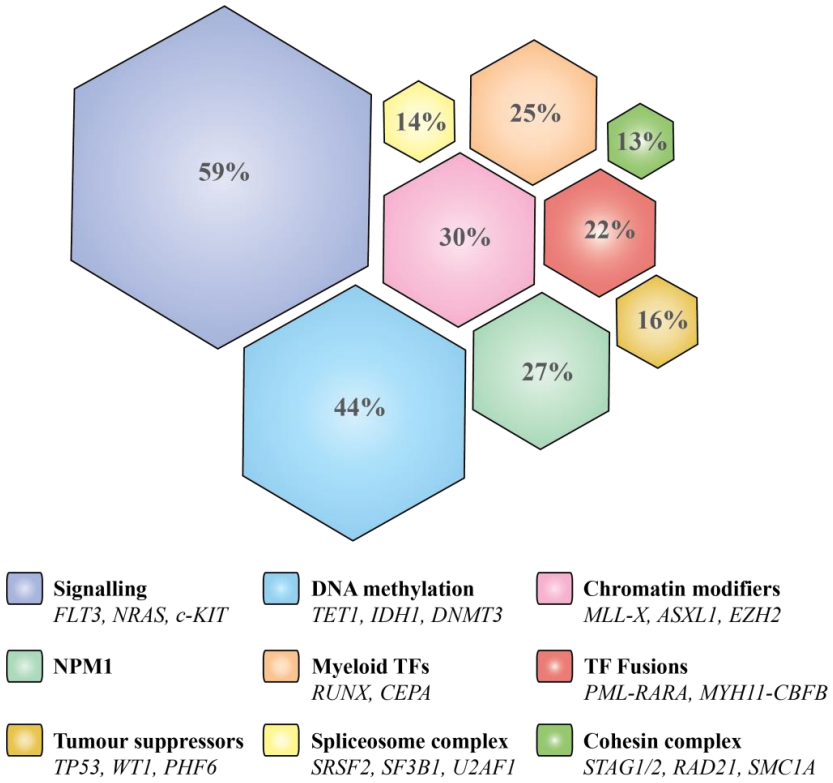

Figure 3. Recurring mutation groups in *de novo* AML and example genes. Gene sequencing studies have identified frequently recurring mutations in *de novo* AML patients that can be categorised into 9 distinct functional subgroups. The 9 groups are ranked with regard to the expected percentage of patients with subgroup related mutations. Example genes from each of the subgroups are listed in coloured boxes relating to each of the 9 categories. Mutations of the *nucleophosmin* gene, *NPM1* occur with such frequency that the gene constitutes its own subgroup. Adapted from (30).

Patterns of co-mutation emerge implying cooperative relationships and mutual exclusivity between genes and even specific mutations (27, 31). For example, mutation of NPM1 is often concurrent with the $NRAS^{G12/13}$ but is not associated with $NRAS^{Q61}$ (29). Mutation of cohesin complex is observed in approximately 10% of AML patients and occur almost exclusively together with *NPM1*, *TET2*, *RUNX1* or *DNMT3A* mutations (32). Co-occurring mutations also impact and inform prognosis (33). *FLT3-ITD* mutation is associated with poor prognosis and survival of this patient group is

significantly worsened if mutations in both *NPM1* and *DNMT3A* are also present (33). Importantly, this reveals that individual mutations, when compared between patients, cannot be ascribed functional equivalence. The unique genomic context of an individual patient will influence the impact of any given mutation. Such a high-resolution understanding of the genomic landscape of AML must be considered for therapy development. Already whole genome and deep sequencing strategies performed on AML patient material are revolutionizing disease categorization and prognostic practice (29). Furthermore, comprehensive genomic analysis intimates a temporal relationship between founder and driver AML mutations implying a clonal evolution of the disease (34).

1.3.2 Clonality and leukemic stem cells

Peter Nowell first proposed the clonal evolution of tumour cells in 1976 (35). The concept has since been validated and described in multiple cancer types with AML serving as a paradigm for investigating clonal evolution in cancer (30). A simplistic but useful model of clonal evolution in AML posits that the process begins with a genomic alteration that confers a proliferation advantage in a single myeloid blast (36). The resulting expansion of the affected cell and its descendants initiates clonal hematopoiesis of variable severity. For transformation into the leukemic state, additional mutations or lesions are required to repress differentiation (36). Informed by this model of leukaemogenesis and disease progression, the concept of a leukemic stem cell (LSC) emerged and was first robustly described in 1997 (37). LSCs are functionally distinct from bulk AML blasts by their increased capacity for self-renewal and ability to initiate malignant haematopoiesis (34). The discovery of LSCs revealed a hierarchical organisation of AML that compliments the model of clonal evolution subject to competition and selection pressures. When understood together, these two concepts (the LSC and subsequent clonal expansion of blasts) serve to explain the genomic complexity observed in many patients at clinical presentation (Figure 1b and c).

Studies continue to annotate and describe leukemogensis and disease progress in greater detail. By isolating peripheral blood cells from AML patients, T cells can be expanded and compared genetically with leukemic blasts form the same patient. Interestingly, in some cases mature non-leukemic T cells contain the DNMT3^{mut} previously speculated to be a founder mutation in AML. Leukemic cells from the same individuals were also positive for the DNMT3^{mut} allele but contained additional lesions absent in the T cells (6). These observations reveal that the DNMT3^{mut} event occurs in an ancestral cell that is capable developing into mature non-malignant hematopoietic cells but also the AML clones present at diagnosis. These ancestral cells containing AML founder mutations (e.g. DNMT3^{mut}, IDH2) have a competitive advantage for repopulating the bone marrow and are referred to as pre-leukemic HSCs. Importantly, it has also been described that pre-leukemic HSCs survive chemotherapy and develop into multiple clones that coexist in an individual patient (38, 39). Furthermore, these insights reveal mechanisms of relapse following perceived remission of patients post chemotherapy (7). Approximately 70% of AML patients who achieve CR will relapse within 5 years (40), further emphasising the importance of these findings.

The developing understanding of leukemogenesis and the genomics of AML has substantial implications for drug development. The contemporary molecular picture of an AML patient at presentation, combined with their unique genetic and clinical history, reveals a multidimensional landscape of opportunities for therapeutic intervention. AML can now be described along a temporal axis with multiple clones existing coincidentally and emerging sequentially. Novel therapies must be assessed for their capacity to impact the spectrum of malignant cell types operating over time to drive disease progression, resistance, and relapse.

1.4 Small molecules as a therapeutic solution in AML

The pursuit of small molecules capable of significantly improving clinical outcomes for AML patients should first consider evidence that such a therapeutic approach is viable.

Acute Promyelocytic Leukaemia (APL)

Acute promyelocytic leukaemia (APL) is a subtype of AML characterised by the *PML-RARA* fusion gene. Reciprocal translocation of chromosomes 15 and 17, results in expression of the PML-RARA fusion protein (41). The retinoic acid receptor alpha (RARA), and its ligand retinoic acid, function to regulate gene expression necessary for the differentiation of haematopoietic cells. When fused with the PML protein, the truncated RARA possesses altered DNA binding and transcriptional activity leading to differentiation arrest. Following a series of *in vitro* observations, in 1988 it was discovered that treatment with all-trans retinoic acid (ATRA), an analogue of vitamin A, could transform clinical prognosis of APL patients (42). When the concentration of retinoic acid exceeds the physiological norms, the ligand constitutively binds RARA portion of the fusion protein, alleviating transcriptional repression. Differentiation is restored and the APL phenotype regresses. The impact of ATRA treatment cannot be understated and APL has been transformed from the most fatal form of AML to the subgroup of the disease with the highest survival rate (43). The identification of arsenic trioxide (ATO) as a synergistic combination partner for ATRA (44) further enhanced clinical response and is capable of curing the vast majority of frontline APL patients, with no need for conventional chemotherapy regimens (45). The success of APL therapy provided an early indication of the clinical potential of molecularly targeted therapy.

Chronic Myeloid Leukaemia (CML)

ATRA therapy in APL exploits the naturally occurring ligand and receptor relationship to repress the oncogenic properties of the PML-RARA fusion protein. Like APL, chronic myeloid leukaemia (CML) is characterised by the presence of an chromosome translocation causing expression of the oncogenic fusion protein, BCR-ABL in immature myeloid cells (46, 47). The *ABL1* gene of chromosome 9 codes for a tyrosine kinase protein that when transcribed in fusion with the *BCR* gene of chromosome 22, generates the constitutively active BCR-ABL kinase (48). CML therapy and clinical prognosis has been revolutionised by small molecule therapy. Imatinib is a tyrosine

kinase inhibitor (TKI) developed by the pharmaceutical company Novartis and approved for CML therapy in 2001 (49, 50). Unlike ATRA, imatinib was developed synthetically by screening small molecules for TKI activity and chemically modifying derivatives of a lead compound (51). By occupying the ATP binding pocket of BCR-ABL, imatinib inhibits phosphorylation mediated signalling culminating in cell death and disease regression (49). For chronic phase CML patients, 8-year survival prior to 2001 ranged from 42-65%, since the introduction of imatinib and its derivatives the life expectancy for CML patients is close to equivalent to the general population (52).

Small molecule strategies in AML

Considering the success of small molecule therapies in APL and CML is useful and relevant for the development of therapeutic strategies in AML. All three conditions are cancers of the bone marrow and blood caused by the transformation of myeloid cells. As such, the coarse features of the microenvironment surrounding the malignant cells are comparable. The efficacy of ATRA and imatinib may imply that curative therapy is achievable in blood cancers if the correct agent or drug combinations are identified. Unlike APL and CML, a particular and recurring genetic lesion does not primarily drive AML. The challenge of developing small molecule agents for the treatment of AML is therefore partially related to overcoming inter and intra patient heterogeneity and the emergence of drug resistance mechanisms (53).

Various strategies have been employed to develop and introduce novel clinical agents for treatment of AML (53, 54). Figure 4 compares alternative models for combating heterogeneity among AML patients.

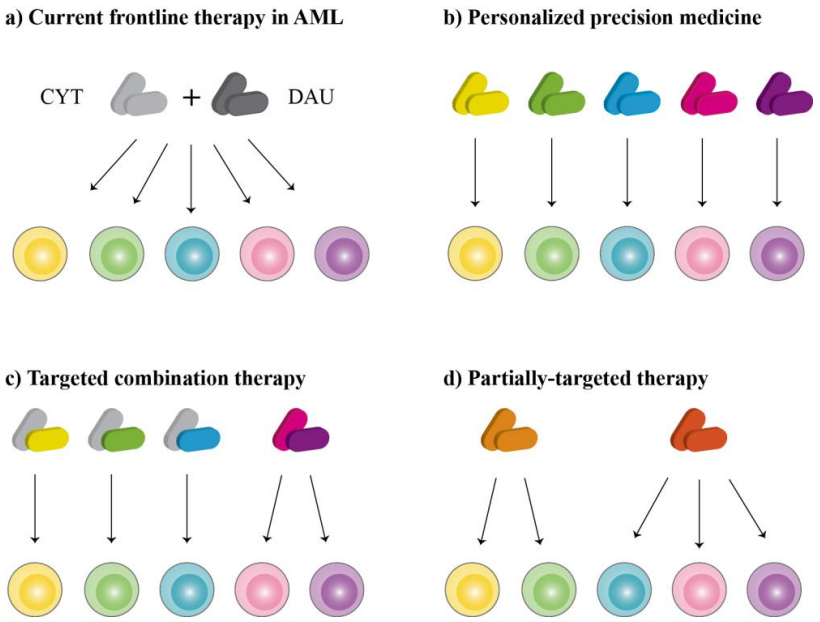

Figure 4. Alternative models of drug therapy for targeting AML heterogeneity. (a) Genetically diverse AML patients receive the same standard chemotherapy regime. CYT = cytarabine, DAU = daunorubicin. **(b)** Genetically diverse patients receive targeted therapy tailored to the specific molecular features of their disease. **(c)** Genetically diverse AML patients receive targeted therapy combined with chemotherapy. Alternatively, targeted therapies are combined to overcome clonality or to achieve synergistic efficacy. **(d)** Genetically diverse patients may be grouped by dependence on cellular processes or shared molecular features. Compounds targeting cellular processes or multiple molecular targets may be capable of treating pooled subgroups of patients despite underlying genetic variance.

Targeted therapies attempt to replicate the success of tyrosine kinase therapy in CML by providing a personalised precision medicine for individual patients or patient subgroups. Small molecules are developed to target specific molecular lesions, such as mutation of the *FLT3* gene. Approximately one third of AML patients are positive for activating mutations of the *FLT3* gene and these patients are associated with poor clinical outcomes (55). Particularly, such patients are prone to relapse following complete remission with standard-of-care therapy (56). A sustained effort over multiple decades has developed a range of compounds to target mutations of the receptor tyrosine kinase, FLT3 (55). Gilteritinib, the most successful agent identified thus far,

is a type 1 FLT3 inhibitor with high selectivity across commonly mutated forms of the protein (internal tandem duplicate, ITD and tyrosine kinase domain, TKI, mutations) (57). In patients carrying a FLT3 mutation with relapsed or refractory AML (R/R-AML) Gilteritinib was associated with higher response rates and significantly increased median survival as compared with standard chemotherapy regimens (Gilteritinib, 9.3 months vs standard chemotherapy 5.6 months) (58). Gilteritinib is now FDA approved for R/R-AML and is currently undergoing clinical trials for use as a frontline therapy combined with standard-of-care regimes or with azacitidine for unfit patients unable to tolerate intense chemotherapy (59). Though developed and presented as targeted FLT-3 inhibitor, it is important to appreciate the agent's multitargeting properties as they likely contribute to efficacy. Gilteritinib targets a range of additional tyrosine kinases but each with varying potency that together reveal a favourable inhibition profile that contributes to the compound's efficacy in AML (59). For example, Gilteritinib's potency for the FLT3-ITD is five times greater than that of the wildtype protein (59). Similarly, the agent has modest potency against AXL, a TKI implicated in AML therapy resistance, but negligible activity towards c-Kit, an important kinase for normal haematopoietic tissue (60). Gilteritinib has selective yet multitargeted activity and early reports suggest the agent can successfully be combined with intensive chemotherapy (61) The agent therefore spans many of the categories of activity outlined in Figure 4. Such range and versatility in activity and targets may represent a hallmark quality required for small molecules to successfully treat AML.

Another example of emerging small molecule therapies with great potential in AML is the combination of BCL-2 inhibitor, venetoclax and demethylating agent, 5-azacytidine. The drug combination was found to synergise in AML cell lines, primary AML cells and preclinical models of the disease (62, 63). As a specific BCL-2 inhibitor, venetoclax represents a targeted therapy that attempts to influence the fundamental cellular process of apoptosis. 5-azacitidine is traditionally characterised as epigenetic regulator through demethylating activity (64). However, by investigating the agent's capacity to synergize with venetoclax, a novel non-epigenetic mechanism of action was revealed whereby 5-azacitidine is able to prime AML cells for apoptosis by induction of the proapoptotic factors NOXA and PUMA (63). A phase 3 clinical

trial of AML patients unsuited for intensive chemotherapy demonstrated the combination's superiority over 5-azacitidine therapy alone and represents a significant milestone towards improving outcomes in this challenging subgroup of patients (65).

1.5 Drug development

Drug discovery is an arduous, expensive and time-consuming process. For 10 anti-cancer compounds developed and approved in the US, between January 1st 2006 and December 31st 2015, the median R&D cost was determined to be 648 million dollars (66). Based on this estimate, the cost of producing a single cancer drug is more than 7 times greater than the cost of launching a state-of-the-art space shuttle into the earth's orbit (67). Industrial drug development on this scale typically comprises multiple phases extending over many years before a compound is approved for clinical practice. Whilst the budget and infrastructure of commercial drug development cannot be replicated in an academic setting, each domain of research activity is continually intersecting. Academic studies inform target discovery and direct collaboration with pharmaceutical companies can greatly accelerate the development and translation of cancer therapies (68, 69). To develop cancer drugs in an exclusively academic environment, alternative cost-saving strategies are required (70). The small molecules explored in this thesis were selected and developed using a variety of distinct but related discovery systems.

1.6 Systems for small molecule identification

1.6.1 Generation of new small molecules

A cross disciplinary approach combining synthetic chemistry and anti-cancer research creates powerful opportunities for small molecule development (71, 72). The generation of novel small molecules is a highly specialized and demanding process requiring particular skills and equipment. In paper I we collaborated with the Bjørsvik Research Group at the Department of Chemistry, University of Bergen, whose research interests include organic synthesis and organometallic chemistry. Organic synthesis

refers to the construction of organic compounds, often through the development of novel synthetic pathways (72). Organometallic chemistry is concerned with compounds or reactions containing carbon - metal bonds (73). Our collaborators developed novel synthetic methods to generate N-heterocyclic carbenes from imidazole precursors (74). An N-heterocyclic carbene (NHC) is a cyclic molecule containing a carbene and at least one nitrogen atom within the carbene ring structure (75). NHC molecules are well suited as ligands for transition metals. Using the methods outlined in Paper I our collaborators produced two novel NHC-silver complexes, NHC-1 and NHC-2 to be investigated as small molecule therapeutics.

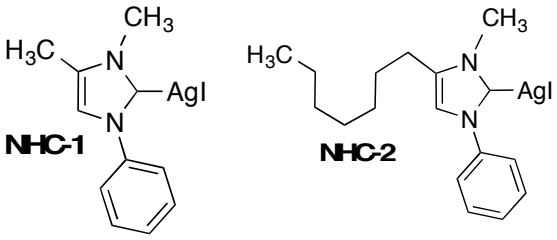

Figure 5. The chemical structure of novel N-heterocyclic carbene – silver complex molecules, NHC-1 and NHC-2. NHC-1 chemical formula: $C_{11}H_{13}AgIN_2$, molecular weight: 408.01 g/mol. NHC-2 chemical formula: $C_{17}H_{25}AgIN_2$, molecular weight: 492.02 g/mol.

Silver has been historically associated with various medicinal properties with studies focusing mostly on antibiotic activity (76). Silver ions (Ag+) are biologically active and interact with proteins, amino acids and cell membranes in both microbial and eukaryotic cells (77). Bacterial resistance to silver ions has been correlated to gene expression and the emergence of specific mutations (78). More recently silver and specifically NHC-silver complexes have been shown to possess cytotoxic activity across a variety of cancer cell lines (79) supporting the rationale of the screening the compounds in leukaemia cell lines. The NHC-1 and NHC-2 molecules investigated vary on the R group on the 4-position of the imidazole ring as illustrated in Figure 9. Investigating such structural analogues enables screening activity to guide novel synthesis strategies and offers a unique and powerful approach to small molecule development.

1.6.2 Reevaluation of industry led targeted therapies

Serdemetan

Industrial drug development typically begins with a target identification phase. The *TP53* gene encoding for the tumour suppressor protein, p53, is the most frequently mutated gene in human cancer (80). Furthermore, in cancers where p53 is infrequently mutated, such as AML, negative regulators suppress the protein's activity (81, 82). Proteins responsible for p53 degradation such as the E3 ubiquitin ligase, human double minute 2 (HDM2), therefore represent a desirable target for cancer drug development (83, 84). Serdemetan (SDM) is a tryptamine derivative isolated from a chemical screen performed by Janssen Pharmaceutical to identify agents capable of restoring p53 activity (85).

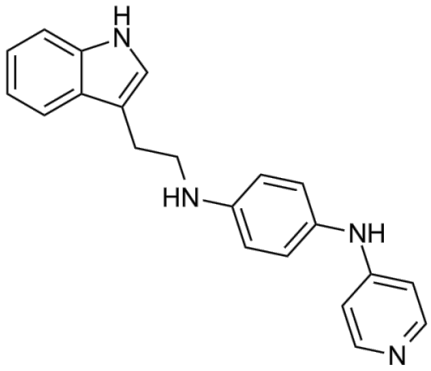

Figure 6. The chemical structure of Serdemetan.
Molecular Formula: $C_{21}H_{20}N_4$, Molecular weight: 328.4 g/mol.

Initially described as an HDM2 inhibitor, the compound was later discovered to exhibit activity independent of the p53-HDM2 regulatory axis (86). Other studies have indicated that SDM targets cholesterol trafficking and HIF1alpha expression, though much of the drugs molecular mechanism remains unclear (87, 88).

Clinical and commercial development of SDM was halted following a phase 1 study in patients with advanced solid tumours published in 2011 (89). In addition to establishing the safety and tolerability of SDM, the trial examined pharmacokinetics and p53

expression in tissues. 71 patients with diverse tumour types were exposed to escalating doses of SDM. Disease stabilization was achieved in 40% of patients, with one breast cancer patient achieving partial response correlating with p53 induction. Despite modest clinical activity, adverse effects limited the success of the trial. QTc prolongation was observed in 14 patients and incidence was associated with SDM treatment in a dose dependent manner (89).

Whilst adverse events appear to have significantly hindered its clinical and commercial interest, migration of SDM into an academic research environment may rejuvenate its therapeutic potential. Further elucidating the mechanism of action and identifying an appropriate disease context or patient sub-group may provide new clinical opportunities. Given that QTc prolongation was deemed dose-dependent, identification of the correct disease context may enable reduced dosing schedules to mitigate the risk of adverse effects. Paper II investigates the potential of SDM for the treatment of AML.

1.6.3 Drug repositioning and drug repurposing

The terms drug repositioning and drug repurposing are often used interchangeably in the field of drug discovery (90). For the purpose of categorising the compounds investigated within this thesis, a distinction will be drawn between the two terms:

Drug repositioning: *"the identification and development of novel applications for a compound* ***within the same disease context****"*.

Drug repurposing: *"the process of redeveloping a clinically approved compound for application in a* ***novel disease context****"*.

When viewed with this distinction, both drug repositioning and repurposing afford unique opportunities for drug discovery and development. Both strategies provide significant pragmatic and financial advantages as compared with conventional commercial drug development as illustrated in Figure 7.

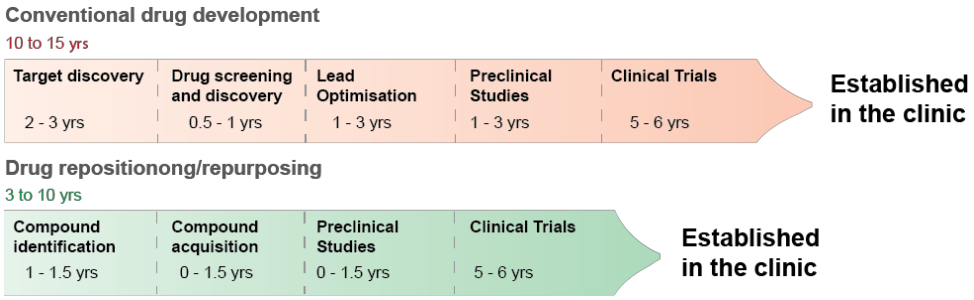

Figure 7. A schematic overview comparing the conventional drug discovery and development timeline with drug repositioning and repurposing. Drug repositioning and repurposing is proposed to offer an expedient alternative to conventional drug discovery by reducing both the number of stages involved in development and the time required for completion of each phase.

Hydroxyurea

Throughout their lifetime, drugs are often subject to repurposing or repositioning resulting in the acquisition of new indications. The arrival of clinically superior agents may relegate compounds to secondary indications. Alternatively, increased understanding of a disease may reveal previously unseen clinical applications for an agent. The evolving role of hydroxyurea (HU) in medicine and cancer therapy serves as a useful example and this compound was investigated in Paper III.

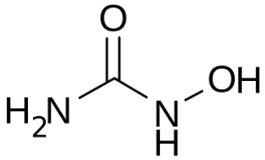

Figure 8. The chemical structure of Hydroxyurea.
Molecular formula: $CH_4N_2O_2$, Molecular weight: 76.06 g/mol.

HU is an antimetabolite that was first investigated for anti-cancer activity in the 1960's (91). HU targets the iron-dependent enzyme ribonucleotide reductase causing a depletion of the nucleotide pool available for dividing cells (92). Additional mechanistic properties have since been attributed to the agent including modulation of DNA methylation and gene expression (93). Over the years HU has been trialled and

incorporated into a broad range of cancer therapy regimes including chronic myeloid leukaemia, head and neck cancers, melanoma and ovarian cancer (94). HU also represents a powerful example of drug repurposing as the compound was granted FDA approval for the treatment of sickle cell anaemia in 1998 and has remained a frontline therapy for the condition (95). The mechanism of action for HU activity in sickle cell anaemia is thought to be multifactorial (96, 97), but disrupted erythropoiesis appears to promote production of fetal haemoglobin reducing sickling of erythrocytes (98). For more than 40 years HU has been administered to AML patients suffering from hyperleukocytosis to achieve efficient cytoreduction prior to induction chemotherapy (99). We hypothesized HU could be repositioned to provide an alternative treatment strategy for AML patients deemed unfit for standard chemotherapy regimens. To succeed HU should be combined with a complimentary drug that can enhance its anti-leukemic properties and enable meaningful disease regression.

Valproic Acid

Valproic acid (VPA) is a branched, short-chained fatty acid first synthesized in 1882 (100). Initially used predominantly as a solvent for organic compounds, VPA was first discovered in 1963 for its anticonvulsive activity (because the solvent itself demonstrated effect) and has since been used to treat multiple neurological disorders including epilepsy, bipolar disorder and schizophrenia among others (101). Subsequently VPA was repurposed as an anticancer agent based on histone deacetylase inhibition (102) and has achieved some clinical impact in the treatment of AML (103).

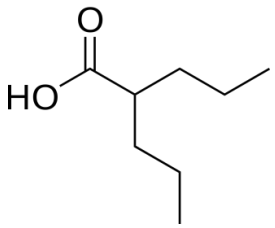

Figure 9. The chemical structure of Valproic Acid.
Molecular formula: $C_8H_{16}O_2$ Molecular weight: 144.211 g/mol.

Transition of euchromatin to heterochromatin is governed by two groups of proteins, histone acetyl transferases (HATs) and histone deacetylases (HDACs). Through epigenetic modification these enzyme families can have a profound influence on gene expression. Restructuring of chromatin occurs in response to histone charge modifications driven by acetylation or deacetylation of lysine residues. Chromatin conformation then dictates recruitment of general transcription machinery and subsequent gene expression (104). Deregulation of HDACs and associated gene expression is commonly observed in AML (105, 106).

VPA specifically targets two of the four classes of HDACs and the resultant effects on cell signaling pathways are extensive (101). In AML cell lines VPA has been shown to decrease expression of oncogene c-Myc, whilst simultaneously increasing cellular accumulation of p21 (107). In addition, VPA decreases expression of anti-apoptotic factors Bcl-2/Bcl-X and enhances FAS dependent induction of apoptosis (101, 108). Interestingly, methylation modulating proteins such as UTX-1 have been identified as potential sensitizers to VPA treatment (109). However, as with many HDACi, the clinical impact of VPA as a monotherapy appears limited (110, 111). The subtle epigenetic regulation incurred by VPA may lend itself well to combinational therapy where VPA can sensitize cells to additional anticancer agents. Modest clinical success has been demonstrated using VPA in combination with ATRA in small populations of high-risk subsets of AML (103, 110, 112). However, in a larger study the addition of VPA and ATRA to standard induction chemotherapy failed to improve overall clinical outcomes (113). Together these observations suggest that VPA has clinical potential for the treatment of AML patients but is likely dependent on identifying suitable

combination partners and appropriate patient subgroups. In paper III we investigate VPA in combination with HU as a therapeutic alternative in AML

1.7 Key molecular pathways and processes

Several molecular pathways and processes have been studied and assayed in the current thesis. A short introduction to each and their relevance in AML is provided below.

Apoptosis: Programmed cell death or apoptosis refers to a mode of cell death characterised by distinct cellular morphology and biochemical signatures (114). A cell's decision to enter apoptosis may be triggered internally or externally causing distinct signalling cascades that converge at the mitochondria and result in the release of proteases responsible for destruction of the cell (115). Profiling of apoptosis associated proteins in AML samples indicates the balance is shifted towards to anti-apoptotic factors (BCL-2, BCL-X and MCL-1) and may be associated with therapy resistance and persistence of minimal residual disease (116, 117). In 1992 it was discovered that phosphotidylserine (PS), a phospholipid which asymmetrically lines the plasma membrane of cells, is flipped to the outer membrane during apoptosis (118). The anti-coagulant protein, AnnexinV binds PS with high affinity and has become a well-established surrogate marker for quantifying apoptotic cells (119). AnnexinV staining combined with nuclear morphology was employed throughout the work performed in the thesis to assess apoptosis. Intriguingly, a variety of conditions in which non-apoptotic cells expose PS are described and may represent novel therapeutic strategies worthy of exploration (120). The studies performed in Paper I indicate a similar tendency is possible in AML cell lines.

Akt: Paper II describes SDM mediated modulation of the signalling protein, Akt. Akt is serine/threonine kinase associated with a variety of cellular processes including cell size, proliferation, metabolism and survival (121). Akt can be expressed as three different isoforms (AKT1, AKT2 and AKT3) whose functions appear to overlap significantly but also exhibit areas of specificity (122). Akt plays a central role in the

PI3K and mTOR signalling pathways that are found to be constitutively activated in AML (123, 124). The downstream consequences of Akt signalling are far reaching and likely context dependent. For instance, Akt mediated phosphorylation of the autophagy protein beclin-1 has been described to inhibit autophagy and stimulate oncogenesis (125). Further studies are required to determine which aspects of Akt signalling, such as its influence on autophagy, hold special relevance for the survival and expansion of AML cells.

Autophagy: Paper II also investigates the cellular process known as autophagy. Autophagy (referring specifically to macro-autophagy in the context of this thesis) is a cellular process common to all eukaryotes involving the regulated degradation of cytoplasmic contents by lysosome activity. Importantly, autophagy enables selective recycling of cellular materials in response to stress such as metabolic depletion or nutrient starvation (126). In cancer cells, both increased autophagic flux and inhibition of autophagy has been associated with disease repression suggesting the process is context dependent (127). In AML, the role of autophagy appears to vary dependent on the molecular context found across heterogenous patient groups but also the clonal composition and blast maturity within individual patients (128). Proteins involved in autophagy are considered essential for the maintenance of normal HSCs (129) and diminished expression of autophagy genes has been associated with particular AML blast phenotypes (130). Conversely, cytoprotective autophagy has been associated with leukaemia initiating stem cells (131). Given the variability but high relevance of autophagy in AML cell fate, the influence of small molecule therapeutics on this complex cellular process is worthy of consideration.

DNA damage and repair: In paper III combination treatment with HU and VPA strongly associated with DNA damage. Various observations implicate the accumulation of DNA damage and dysfunctional repair systems in AML pathogenesis and disease progression (132). Approximately 50-60 % of patients carry one or more cytogenetic abnormalities (133). Furthermore, therapy related AML (t-AML) in patients treated with DNA damaging chemotherapeutics or radiation therapy is increasing and has substantially poorer prognosis than *de novo* AML (134).

Nevertheless, DNA double strand breaks (DSB) represent lethal lesions in DNA capable of inducing apoptosis in cancer cells and repair mechanisms are thus regarded as an important therapeutic target (135). Two key mechanisms exist for repairing DSB, homologous recombination and non-homologous end joining, and both pathways have been described as abnormal or dysregulated in AML (136). A better appreciation of how small molecule drug treatments can damage DNA or influence damage repair systems may be critical for enhancing therapeutic effects in the treatment if AML.

2. Aims of the study

Therapy development for AML has been the subject of intense research for well over 50 years. This effort has transformed our understanding of the disease from both a clinical and molecular perspective. Until recently standard-of-care therapy for AML patients remains largely unchanged from that which was established in 1970's. A series of new approvals since 2017 has begun to chart a path toward significantly improved survival. Nevertheless, the repertoire of effective agents needs to be expanded further and establishing therapy alternatives for patients deemed unsuited to intensive chemotherapy remains a challenge. The aim of this study was to contribute to the discovery and development of small molecules and expand the therapeutic alternatives for AML patients.

Specific aims of the thesis:

1) To support the development of newly synthesized small molecules (Ag-N-Heterocyclic complexes) and assess their cytotoxic activity in AML cell lines.
2) To revaluate the industry developed small molecule, serdemetan, for the treatment of AML and discover novel mechanistic properties associated with the agent's activity
3) To reposition the approved anti-leukemic agent hydroxyurea and repurpose the anti-convulsive agent valproic acid, as a novel combination therapy and evaluate their therapeutic synergy in models of AML

3. Material and methodology considerations

3.1 Source of compounds, preparation and biological concentrations

Valproic acid (VPA): The stocks used for experiments conducted in this thesis were derived from sodium valproate 100 mg/ml solution for injection. The clinical/commercial preparation, Orfiril Injeksjonsvæske (Desitin Arzneimittel GmbH), was sourced from the Haukeland University Hospital, Bergen. In solution, sodium valproate is stable for 3 years unopened. Once opened, aliquots were generated and stored at 4 degrees. No indications of degradation or instability were observed. For *in vitro* studies VPA was diluted directly into cell culture medium, for *in vivo* dosing of animal models' syringes were drawn directly from the 100 mg/ml stock solution. The elimination half-life of VPA varies dependent on age and the presence of other medicines but as a monotherapy ranges from 10 – 20 hrs in adults (137, 138). Serum concentrations of VPA in AML patients varies depending on dosage and frequency of administration though typically ranges from 0.3 – 0.9 mM (113).

Hydroxyurea (HU): Hydroxyurea, 98%, powder was purchased (Sigma Aldrich, Cat. No. H8627) and stored at 4°C. When prepared for *in vitro* studies the powder was first dissolved in water and further diluted directly in cell culture medium. For *in vivo* dosing the compound was dissolved in saline to a concentration of 200 mg/mL. Due to the instability of the compound in aqueous solutions, new preparations were generated daily for all experiments. The elimination half-life of HU is relatively short, 2 – 4 hrs in adults and children, though the compound benefits from strong bioavailability (139). Though technically challenging to determine, the plasma concentration of HU in adult patients can be estimated to range from 0.2 – 1 mM depending on the dose received (140).

Serdemetan (SDM): Serdemetan (also referred to as JNJ-26854165) was provided as a purified powder by Janssen Pharmaceuticals (Beerse, Belgium) and kept at 4°C for long-term storage. For *in vitro* studies the powder was dissolved in DMSO to generate stock solutions for long-term storage at -80°C. Further dilution of stock solutions was

performed directly in cell culture medium. For animal studies SDM was formulated in 10% hydroxy-β-cyclodextrin. Stock solutions for animal studies were stored at 4°C for the duration of dosing cycles. The plasma concentration of SDM in patients during a phase 1 clinical trial ranged from 3 – 7 μM (89). Maximal concentration was achieved 2 – 4 hours following administration and levels were reduced to less than half of peak values after 24 hrs (89).

4-alkylated silver-M-Heterocyclic (NHC) complexes 1 and 2: The novel small molecule Ag-NHC complexes, NHC-1 and NHC-2, were developed and synthesized by the Bjørsvik Research group at the Department of Chemistry, University of Bergen. Synthesis of the molecules concluded with precipitation of crystalline solids that were provided for *in vitro* screening studies. NHC complexes were stored at 4°C in solid forms and dissolved in DMSO to generate stock solutions for long-term storage at -80°C. Further dilution of stock solutions was performed directly in cell culture medium. The half-life of these agents remains to be determined, as do the clinically and biologically relevant concentrations.

3.2 Cell lines and primary patient material

Two sources of AML cells were used throughout the studies performed within the thesis: primary patient material and cell lines.

The primary AML patient cells studied within the thesis are collected directly from the peripheral blood or bone marrow of patients diagnosed and treated at Haukeland University Hospital. Following sample collection, mononuclear cells are isolated by density gradient separation and cryopreserved in liquid nitrogen or stored at -150°C. Primary AML cells represent a precious resource for assessing drug efficacy in a highly relevant cellular context. If compounds can be screened in a sufficient number of primary AML samples patterns of sensitivity and resistance may emerge revealing patient subgroups of particular interest for a given therapeutic agent. Furthermore, screening performed in parallel with high resolution molecular characterisation of primary material may facilitate the identification of biomarkers for clinical translation.

Nevertheless, a variety of challenges and limitations are associated with primary cell culture experiments. Cells isolated from patients are not accustomed to *in vitro* cell culture conditions. The viability and proliferative capacity of cells varies greatly across patients and even across sampling time-points of individual patients. Such variations are a likely source of bias in sample populations as samples which better tolerate *in vitro* culturing are more often included in studies (141). Reproducibility may also be compromised when working with primary cells if insufficient material is available generate experimental replicates. Variation is also generated in how samples are processed when initially collected and further cultured *in vitro*. Though attempts have been made to determine the optimal culture medium growth factors and supplements for the culture of primary AML cells (142) there is no standardised protocol across research institutes. Some of the challenges associated with primary cell cultures can be addressed through the use of cell lines.

Cell lines are commercially available monoclonal cell cultures. Originally derived from primary material, cell lines are immortalized, either spontaneously in culture or through genetic manipulation, and characterised by unlimited proliferative potential and the ability to override mechanisms of senescence. Cell lines can therefore be maintained in long-term cultures allowing experiments to be optimised reproduced and replicated by other research institutes in standardised culture conditions. Additionally, the stability of culture conditions permits the manipulation of cell lines. Genetic modification may involve transduction of cells to express a gene of interest or conversely to abolish expression of a target protein. Long-term culturing also enables the establishment drug resistant cell lines (143). Cells are cultured in gradually increasing concentrations of a drug of interest eventually yielding drug-adapted cell lines resistant to high-dose treatments. Subsequent molecular profiling of such cell lines may reveal mechanisms of resistant that parallel disease progression in vivo.

Despite offering powerful research tools it is not clear to what extent AML cell lines accurately reflect the cells driving the disease *in vivo*. Furthermore, despite the large number of cell lines available, the disease heterogeneity of AML cannot be captured using these models. The stability and reproducibility of cell lines has also been

scrutinized with significant concerns regarding over-passaging, authentication and genetic drift within cultures (144, 145).

3.3 Screening strategies for small molecule identification

Evaluating which compounds were worthy of further investigation was a critical phase in the development of the work performed in the thesis. The screening and selection of small molecules presents challenges relating to both methodological considerations and experimental design.

Screening strategies to identify small molecules with anti-cancer activity generally follow one of two strategies:

1) High-throughput screening of extensive drug libraries using simple assays to identify candidates worthy of further investigation.
2) Selective screening of particular compounds based on a rationale relating to properties of the drugs and their suitability to a particular disease context.

High-throughput screening on a large scale requires automation and bioinformatic expertise to generate and process large sets of data (146). Cell lines or primary cells are screened in 96 or 384 well plates using simple absorbance-based cell viability assays. High-throughput strategies are highly efficient and powerful but are inflexible and the data produced for any single agent maybe not be robust and demands subsequent validation. Elaborations of the high-throughput screening strategy include flow cytometry-based assays to identify agents targeting specific cell subpopulations or combination screening to overcome resistance mechanisms (147, 148). Drug screening may also be combined with RNA interference or CRISPR-based genetic screens to discover genes or pathways revealing cancer cell vulnerabilities and novel druggable targets (149, 150).

Selective screening of fewer compounds enables selection of assays well suited for the compounds and cell types being screened. As previously described the *in vitro* proliferative capacity of primary AML samples varies significantly across patients

and therefore it can be important to select screening assays that control for such variation or at least avoid amplifying the bias. Selective screening also enables sophisticated assay readout when particular drug properties or cellular phenotypes are to be investigated.

The work performed in this thesis attempted to find a balance between the screening strategies described above. Paper III provides multiple examples of how methodological consideration and study design may influence project development. Hydroxyurea (HU) is a potent inhibitor of cell proliferation but requires higher doses to incite cell death. If screening assays capture only cell death induction the antiproliferative properties of HU may be overlooked. Cell cycle analysis was performed in order to assess the antiproliferative capacity of HU in combination with valproic acid (VPA) and provided insights that directed further experiments to delineate the combinations mechanism of action in AML cells. Interestingly, selection of HU and VPA as combination partners was not a product of extensive screening but examination of existing literature suggesting HDAC inhibitors can enhance the effect of antimetabolites such as HU. Interestingly, later work performed during the thesis validated the strategy in a high-throughput screening format. An experiment was performed to identify additional combination partners for VPA in collaboration with the Institute for Molecular Medicine Finland (FIMM). Utilising the drug sensitivity and resistance testing (DSRT) platform established at FIMM the AML cell line MV4-11 was screened against 461 small molecules in the presence and absence of clinically VPA at a clinically relevant concentration (0.6 mM) Dose-response curves were generated for all compounds under each condition. Screened compounds were ranked according to the enhancement of effect of VPA as is illustrated in Figure 10. HU efficacy was determined to be significantly enhanced in the presence of VPA and ranked 12th among the of 461 molecules analysed. The compatibility of HU and VPA for the treatment of AML cells was therefore independently identified across two screening strategies explored during the completion of the thesis.

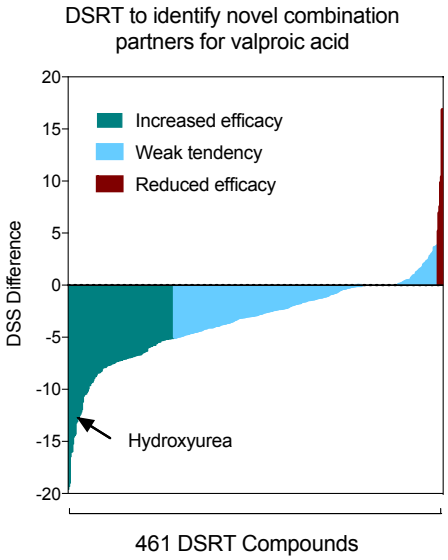

Figure 10. Drug sensitivity and resistance testing (DSRT) to identify small molecules with enhanced efficacy in the presence of valproic acid (VPA). MV4-11 cells were screened against 461 DSRT compounds across a range of 5 concentrations for 72 hours in the presence and absence of valproic acid at a concentration of 0.6 mM. Dose-response curves were generated using integrated Cell Titer Glo viability assay (Promega). Drug sensitivity scores (DSS) were generated based on dose response curves and compared for each compound screened +/- VPA. A difference in DSS score greater than 5 was considered to indicate a significant tendency for increased or reduced efficacy. Line and arrow head indicate the ranked position of hydroxyurea.

The work performed in this thesis demonstrates that drug screening can be achieved utilising a variety of methodological and design strategies. Screening represents a critical stage in drug development and benefits from multi-parameter analysis and appropriate assay selection.

3.4 Preclinical models of leukaemia

As illustrated previously in Figure 7, preclinical studies represent a critical phase common to both conventional and alternative approaches to cancer drug development. Preclinical studies utilise animal models to assess toxicity and drug efficacy but can

also reveal novel insights into disease pathophysiology not possible from *in vitro* studies. Successful preclinical studies are generally considered a perquisite for progression to clinical trials. Figure 11 provides an overview of the preclinical models of AML that were employed in this work.

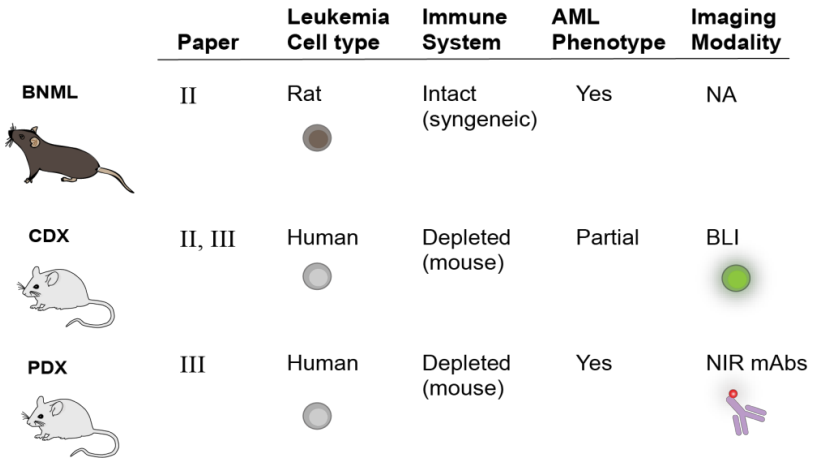

Figure 11. Summary of the preclinical models used to assess *in vivo* drug efficacy. The BNML (Brown Norway Myelocytic Leukemia) model is a syngeneic rat model of leukaemia in which disease pathology reflects AML in humans. CDX (cell line derived xenograft) and PDX (patient cell derived xenograft) models are generated by inoculation of human leukaemia cells into NOD/SCID IL2rγ^{null} (NSG) mice. Due to their highly compromised immune system, NSG mice facilitate engraftment and expansion of AML cells. Disease progression and characteristics vary dependent on cell line or patient cells employed. NA, not available; BLI, bioluminescent imaging; NIR, near-infrared; mABs, monoclonal antibodies.

BNML: The brown Norway myelocytic leukemia (BNML) model was first described in 1971 and was generated by infusing BN rats with the tumour initiator agent, 7,12-Dimethylbenz[a]anthracene (DABA) (151). Importantly, leukemic cells isolated from the spleen of the animals could be transplanted into new BN hosts and recapitulate the disease. The disease phenotype was characterized as a myelocytic leukemia (152, 153) and isolated BNML cells were highly comparable to primary AML cells in colony formation assays (154). As a syngeneic model the BNML rats have provided a powerful tool for investigating AML progression in the context of normal

hematopoiesis. Significant insights into chemotherapy efficacy, toxicity and resistance were achieved using BNML rats (155, 156) and the model is considered highly relevant for AML therapy development. These properties made the BNML model well matched for testing serdemetan (Paper II), as the study aimed to illustrate the agent's suitability for AML but also to allay concerns over toxicity. The BNML requires modernization with deep mutational characterization to demonstrate its relevance to human AMLs on a molecular level.

Xenograft models: Cell line-derived xenograft (CDX) models of cancer are generated from implantation or engraftment of *in vitro* cultured cell lines into suitable mouse strains for *in vivo* studies. In patient-derived cell xenografts (PDX) cells are sourced from primary patient material and are typically implanted directly into mouse models without prior *in vitro* culturing. Xenograft models of AML have been revolutionized by the development of increasingly immunocompromised mouse strains facilitating greater rates of AML cell engraftment (157). Figure 12 summarizes the key mouse strains used to develop AML models.

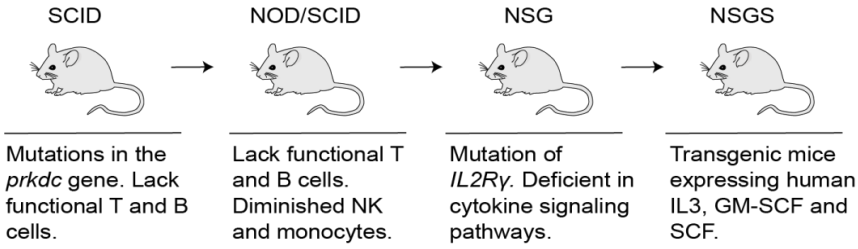

Figure 12. Summary of the key mouse strains used for the development of AML xenograft models. The strains are described from left to right in order of increasingly immunocompromised and corresponding enhancement of AML cell engraftment. SCID, severe combined immunodeficient; NOD, non-obese diabetic; NSG, NOD/SCID IL2Rγ^{null}; NSGS, designated trademark name for NSG transgenic mice expressing the listed human growth factors, IL3, GM-CSF and SCF.

Both CDX and PDX models employed in this thesis used the NSG strain of mice for engraftment. AML cells are injected intravenously and when successful will home to the bone marrow and spleen and proliferate to generate a model of leukaemia. Disease

latency, phenotype and responsiveness to therapy varies across the cell lines or primary material employed. PDX models are demanding to generate as engraftment rates across primary cells are generally low. Furthermore, cells must be passaged through consecutive animals until disease latency and phenotype are stabilized. Cell material harvested from animals should be sequenced to confirm the molecular characteristics of the original patient cells are intact. Importantly, PDX models generally better reflect the AML disease phenotype with cells primarily developing in the bone marrow and spleen. CDX models may be compromised by erratic metastasis to secondary tissues generating less relevant disease phenotypes.

4. Summary of papers

4.1 Paper I

In paper I we supported the development of novel small molecules and conducted a preliminary investigation to assess their cytotoxic properties in AML cell lines. Backbone-alkylated imidazoles were generated and complexed with silver using a novel multi-step synthetic pathway to produce the putative metallodrugs, NHC-1 and NHC-2. The complexes vary only at position 4 of the imidazole ring backbone where NHC-1 carries a methyl group and NHC-2 a heptyl group. The cytotoxic properties of both complexes were compared in the AML cell lines, HL-60 and MOLM-13. NHC-2 was revealed to be the most potent of the complexes and we speculate that the presence of the heptyl side group may facilitate more efficient release of silver following uptake to cells. Additionally, we observed that for both complexes that induction of cell death induction rapidly and was associated with apoptotic nuclear morphology. Interestingly, we also observed increased phosphatidylserine expression in HL-60 cells treated with the silver NHC complexes as compared with cytarabine.

4.2 Paper II

In paper II we investigated the tryptamine derivative serdemetan to assess its suitability for further development and use in AML. Initially developed as an inhibitor of MDM2 we first screened the compound in a collection of AML cell lines revealing that unlike typical MDM2 inhibitors the agent's activity does not appear to be strongly influenced by the expression of WT p53. SDM activity was then compared with an established MDM2 inhibitor, Nutlin-3, across a range of primary AML samples revealing similar though not identical activity profiles in regard to induction of apoptosis and inhibition of proliferation. Preclinical studies in BNML rats and MOLM-13 mice xenografts were performed revealing the agent's anti-leukemic activity was maintained *in vivo*. Interestingly, in these models SDM performed favourably as compared to Nutlin-3 and daunorubicin and exhibited efficacy similar to that of cytarabine. Finally, we wished to identify novel properties associated with SDMs mechanism of action. In MOLM-13

cells SDM treatment was associated with upregulation of autophagy markers and depletion Akt1 expression.

4.3 Paper III

In paper III we aimed to reposition Hydroxyurea (HU) and Valproic acid (VPA) as a new combination therapy worthy of further development in AML. HU and VPA synergised to induce cell death in diverse AML cell line and combination efficiency was at least partially governed by p53 status. In recovery assays removal of drug treatment after 72 hours exposure indicated that both wildtype p53 expression and the presence of both agents was required to generate an enduring anti-proliferative effect. Analysis of cell cycle in OCI-AML3 cells showed combination treatment induced comprehensive S-phase arrest and that the sequence in which cells were exposed to each of the compounds was decisive for induction of apoptosis. Combination treatment was strongly associated with expression γH2AX indicating DNA double strand breaks and depletion of the key homologous recombination protein, RAD51. Finally, we confirmed that the superior efficiency of the combination was maintained in primary AML samples and also in preclinical models of the disease, including an OCI-AML3 xenograft and an aggressive patient derived xenograft model.

5. Discussion

5.1 Evaluation of compound identification strategies

Various strategies were employed to identify small molecules worthy of further development for the treatment of AML, each with their own strengths and limitations.

Generation of new small molecules: To produce novel small molecules in an academic setting mounts a variety of challenges. Our approach capitalised on the expertise of our collaborators in organometallic chemistry and intuitions regarding the therapeutic properties of compounds containing silver. In contrast to commercially acquired agents, studies with the novel silver complexes required rigorous and consistent quality control studies to monitor batch purity in regard to both chemical structure and biological activity. Another limiting factor when working with internally developed compounds relates to scale of production and yield of end products. The synthesis procedure associated with NHC-1 and NHC-2 produced plentiful amounts for conducting *in vitro* studies. However, should either of the drugs (or their derivatives) be developed further to preclinical testing a significant expansion of product yield will be required to facilitate *in vivo* studies. A crucial advantage of producing novel small molecules internally is the ability to further modify and optimise hit-compounds. In a follow up study to Paper I our collaborators generated additional complexes with alterative variations on the imidazole ring to generate a small chemical library (158). Simple structure – activity studies guided development of an improved ligand structure that was able to significantly enhance the potency of the silver drugs (158).

Re-evaluating industry led targeted therapies: From January 2000 to September 2020 a total of 167 targeted agents have reached phase II clinical trials for AML (54). Of these compounds only 8 achieved FDA approval and many more are discarded at phase I. The high volume and low success rate of targeted therapies developed for AML and cancer in general provides a large and diverse pool of agents that may be revaluated. The investigation of serdemetan (SDM) in paper II benefited greatly from its industry led development and associated academic studies. The decision to examine autophagy

in cells treated with SDM was motivated by data shared from the Janssen Pharmaceutical internal drug discovery program indicting the agent was lysosomotrophic (data not shown). Preclinical testing is generally a perquisite for progression to clinical trials and as such industry developed agents are well tested in animal models and these studies can guide and expediate preclinical work involved in redevelopment studies. SDM was tested extensively in a variety of tumour xenograft models (159), and these studies served as a reference point for designing the preclinical work performed in paper II.

Redeveloping industry led small molecules also presents significant challenges. Compounds fail to progress through clinical trials for a variety of reasons often related to efficacy and toxicity but also due to financial or bureaucratic challenges (160). The unique obstacles of a particular agent must be carefully considered and overcome during redevelopment. In the case of SDM it seems clinical development was suspended primarily due to dose dependent QTc prolongation observed during a phase I clinical trial in solid tumours (89). Our approach to circumvent this challenge was to identify a new disease context, AML, in which lower doses may be sufficient for efficacy. It would have been valuable to investigate this toxic effect directly in our study. QTc toxicity is commonly associated with anticancer targeted therapy (161, 162). Many targeted therapies appear to disrupt currents of cardiac action potential by inhibiting the hERG subunit of potassium ion channels (161). In vitro systems have been developed to determine the IC_{50} for hERG inhibition (161, 163) and assessing SDM in such an assay would likely aid future development. The concentration of SDM required to cause significant hERG inhibition could be compared to the concentrations expected to achieve anti-leukemic activity in patients as extrapolated from the preclinical studies in paper II.

Drug repositioning and drug repurposing: Drug repositioning and repurposing offer a variety of advantages and challenges when selecting small molecules for investigation and development. The challenges associated with drug repurposing, in particular, were encountered in work performed adjacent to the studies described in the thesis. Our screening studies identified the antipsychotic agent, chlorpromazine, and the

antimalarial agent, quinacrine, as candidates for repurposing in AML. Chlorpromazine's capacity to affect the central nervous system represent a significant hurdle for translation to the setting of AML. To overcome this challenge, we encapsulated the drug in biodegradable nanoparticle carriers to prevent its crossing the blood brain barrier whilst maintaining antileukemic activity (164).

In the case of repositioning hydroxyurea, as described in this thesis, an extensive body literature already exists assessing the agents' activity in AML in both clinical (165-167) and basic research settings (168, 169). This established literature in the relevant disease context greatly supported the design and execution of the experiments performed in Paper III. Similarly, utility of valproic acid as an AML therapy has been investigated extensively in basic research, preclinical and clinical settings since its original repurposing from an anticonvulsant to a cancer drug (102, 170). The robust anti-leukemic characterisation of HU and VPA helped guide mechanistic studies in Paper III. The capacity for HU to induce DNA double strand breaks is well described in various cancer types (94), whilst the HDAC inhibition activity of VPA is similarly well established (100, 102). Furthermore, the potential for HDAC inhibitors to amplify genotoxic therapies appears to be a class effect for these compounds (171, 172) and encouraged our decision to investigate DNA damage and repair in Paper III. This observation regarding HDAC inhibitors also highlights a potential limitation associated with our drug repurposing approach in that there may exist alternative HDAC inhibitors that are superior in their ability to enhance the effect of HU. Several novel HDAC inhibitors, such as vorinostat and pracinostat, have undergone or are currently enrolled in clinical trials for AML and could have been screened together with HU in paper III (105). However, the combination of HU and VPA has already been tested clinically in single AML patients and appears well tolerated (173), thus emphasising the advantage of repurposing/repositioning, as these data would likely accelerate the future clinical development of the combination.

5.2 Evaluation of compound development strategies

Small molecule development both industrial and academic settings if often tightly associated with target specification and biomarker discovery. Each of the strategies employed to select and develop small molecules in this thesis provided unique challenges in this regard.

Challenges associated with target specification:

In paper I we generated entirely novel synthetic compounds, NHC-1 and NHC-2, as such, target specification acquires a broader definition. Although considered beyond the scope of the narrative in paper I, pilot experiments were performed to investigate whether the cytotoxic activity could be attributed to silver, the imidazole ligand, or the combination as a complex. Additionally, it was important to assess whether the cytotoxic properties of the agents are generalised or specific to cancer or in this case leukaemia cells. Preliminary results investigating these questions indicate that silver is driving the cytotoxic activity and that normal peripheral blood mononuclear cells are more resistant to NHC-2 than the HL-60 cell line (Figure 13A + B).

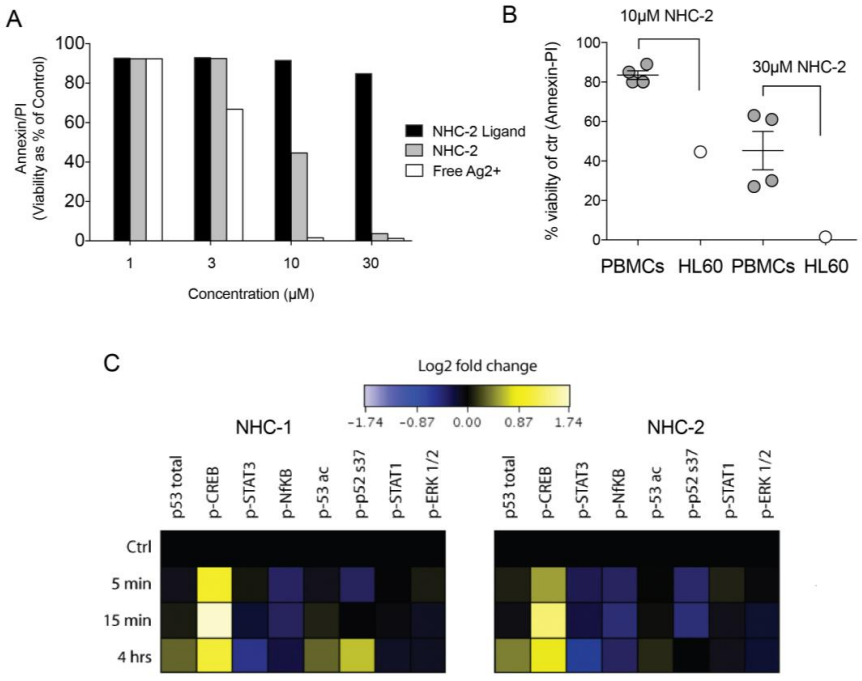

Figure 13. Investigating NHC-Ag drugs ligand activity, cytotoxic specificity, and putative molecular targets. (a) Preliminary data demonstrating induction of apoptosis and cell death in HL-60 cells as determined by annexin/PI staining of cells treated for 24 hrs with a range of concentrations of the NHC-2 ligand in the absence of silver (NHC-2 ligand), complexed with silver (NHC-2) and free silver (Ag2+). **(b)** Induction of apoptosis by NHC-2 treatment for 24 hrs at 10 and 30 μM in PBMCs (peripheral blood mononuclear cells) and compared to HL-60 cells. **(c)** Heatmaps illustrating signalling pathway response following short term exposure to NHC-1 and NHC-2 in MOLM-13 cells.

As we begin to understand the leukemic specificity and active components of the new NHC-Ag molecules future studies should attempt to define molecular targets. A target "fishing" pilot study was performed during the thesis period using flow cytometry to assess the phosphorylation status in key AML signalling proteins, as well as expression and acetylation of p53, following short term exposure to both NHC-1 and NHC-2 (Figure 13C). The experiment indicated a substantial increase in phosphorylated CREB (cAMP response element binding protein) that may be related to the mechanism of action for the silver drugs. Interestingly, in their follow up study our collaborators

speculated that NHC-Ag molecules may intercept or disrupt cellular redox homeostasis via the thioredoxin system. The thioredoxin system has recently been implicated in tumour development (174) and down regulation of the thioredxin-1 has been associated with phosphorylation of CREB (175). Both these pathways should be examined in future studies aiming to specify the target of NHC-Ag compounds.

In paper II we describe novel effects associated with SDM treatment which likely contribute to the molecules anti-leukemic activity. An increase in key autophagy markers and reduction of Akt1 expression was observed in MOLM-13 cells. Paper II could be improved by functional studies to determine whether reduction of Akt1 expression is necessary for autophagy induction and cell death. During the work lentiviral vectors were constructed produce MOLM-13 cells expressing myristolyated, and therefore constitutively activated, Akt1 (176). This experiment aimed to determine whether elevating and sustaining Akt1 activation could protect MOLM-13 cells from SDM induced cell death. Though transduction was successful the MOLM-13 cells seemed to poorly tolerate the transduction and cultures could not be expanded to perform the experiment. Interestingly such observations have been reported in other model systems indicating excessive stimulation of Akt proteins can inhibit proliferation and induce cell death or senescence (177). To resolve this issue alternative cell lines could be tested to see if they better tolerate constitutively activated Akt1 or inducible expression vectors could be designed to adjust myristolyated Akt1 expression to an acceptable level for MOLM-13 cells (178).

In paper III our attempts to define the targets of HU and VPA were focused on understanding the molecular interaction capable of generating their synergistic induction of apoptosis and cell cycle arrest. Phosphorylation at serine 139 of the histone protein H2AX (γH2AX) is a well-established and sensitive marker for DNA double strand breaks (179). In paper III western blotting showed dramatically increased γH2AX in OCI-AML3 cells following 24 hrs combination treatment, whilst single agent treatments showed little no increase as compared with control cells. This observation correlates strongly with the synergistic induction of cell death in AML cell lines but also the combination efficacy in the OCI-AML3 xenograft model where

monotherapies failed to prolong survival over control animals, while survival was significantly increased in combination group animals. Paper III would be strengthened by validating the γH2AX observations by performing experiments to detect DNA double strand breaks directly, such as the comet or STRIDE assays (180, 181).

Paper III may also have benefited from closer investigation of the precise targets of HU and VPA individually. Ribonucleotide reductase is considered the primary target of HU (168) and could have been studied directly in AML samples by assaying subunits of the enzyme or indirectly by monitoring the nucleotide pool of cells exposed to HU (182, 183). Interestingly, a recent study revealed the capacity for HU to suppress the activity of the dNTPase SAMHD1 thus enhancing the sensitivity of AML cells to cytarabine (169). This study illustrates that additional targets modulating HU activity may yet be uncovered. For VPA we primarily attribute its capacity to synergize with HU to its ability reduce Rad51 expression and nuclear foci formation. Further studies are required to define the precise mechanisms through which VPA's targets Rad51 and other DNA repair proteins. Gene expression studies performed in prostate cancer cell lines suggest VPA may downregulate Rad51, Chk1 and Brca1 at the transcriptional level via the transcription factor E2F1 (172). Interestingly, a study published in 2017 cited paper III and aimed to determine more specifically the mechanism by which VPA sensitises cancer cells to HU treatment (184). Using the MCF7 breast cancer cell line the authors discovered that HU induced DNA DSB activate homologous recombination (HR) via phosphorylation of replication protein A2 (RPA2). VPA was shown to disrupt HU mediated activation of RPA2 and its interaction with Rad51 resulting in synthetic lethality (184). Importantly, the study was performed using clinically relevant concentrations of HU and VPA that are achievable in AML patients, further encouraging clinical development of the combination.

Identifying biomarkers and sensitive patient subgroups:

Clearly defining the molecular targets of small molecules supports the identification of biomarkers and sensitive patient subgroups. In papers II and III a variety of primary AML cells were screened to determine sensitivity to SDM and the combination of HU

and VPA. In both studies a range of response was observed across patients though no significant correlations were observed in relation to the available clinical and biological characteristics. In the case of paper II patients could be stratified by expression or activation of Akt1 or autophagy markers to determine whether these pathways can predict sensitivity to SDM treatment. Flow cytometric analysis of the PI3K-AKT-mTOR pathway activation has successfully been used to assess heterogeneity across AML patients but also within individual patient samples capturing clonal heterogeneity (124). A similar approach could be performed to determine the autophagy flux across AML patients screened with SDM. Indeed, such methodologies have already been performed in AML cell lines and patient samples identifying a sub population CD34+ AML blasts with low levels of reactive oxygen species that appear to have a higher dependency on autophagy (185).

In paper III comparison of cell lines expressing WT or mutated/null p53 provided a strong indication WT p53 expression is necessary for HU and VPA mediated induction of cell death. As such, WT p53 status can serve as an initial biomarker for patients suitable for the combination therapy. However, given the majority of AML patients carry WT p53, additional biomarkers related to DNA damage and repair would be beneficial for identification of sensitive patients. The nuclear deacetylase, SIRT6 appears to play an important role in maintaining genome stability in various cancer types (186). Furthermore, it has been shown that SIRT6 expression is variable across AML patients and that the enzymes' activity negatively correlates with sensitivity to DNA damaging therapies (187). SIRT6 activity could be assessed in AML patients screened with HU and VPA combination therapy to explore whether the protein might serve as a valuable biomarker. Such studies, together with additional biomarker candidates should be incorporated into the development of phase 1 clinical trials for the combination therapy.

In paper I we generated entirely novel synthetic compounds, NHC-1 and NHC-2. The data included in the article aims to provide a basic and preliminary characterisation of cytotoxic properties of each agent. Nevertheless, the observation that the TP53 null HL-60 cell line had a lower IC50 for both agents as compared with TP53 wild type

MOLM-13 indicates that a range of responses would likely be obtained if the agents were screened across more cell lines or primary patient samples. Such variations in response can provide a starting point for identifying biomarkers and subgroups of sensitive patients.

5.3 Evaluating preclinical modelling strategies

Multiple preclinical animal models were used to investigate the small molecules studied in papers II and III. In each case the selected models successfully reflected efficacy observed in *in vitro* screening. Importantly *in vivo* animal studies confirmed that serdemetan, HU and VPA were well tolerated and did not reveal unexpected toxicities.

In paper II the syngeneic BNML rat model and a MOLM-13 xenograft model were used to assess the efficacy of serdemetan compared to other anti-leukemic agents (cytarabine, daunorubicin, idarubicin and nutlin-3). In both models two distinct dosing schedules were investigated for serdemetan, low dose and daily treatment vs. weekly or biweekly dosing with a higher dose. Interestingly, in both models daily dosing was observed to be superior in prolonging survival. This finding illustrates how preclinical modelling can inform and guide the design of clinical trials. Despite its extensive history of use in developing AML therapy (155, 156), the BNML rat model is not well characterised in regard to mutations or genomic abnormalities. Paper II would benefit greatly from molecular characterisation of the BNML cells driving the disease as this may provide further insights into serdemetan's mechanism of action. In contrast, the cell line xenograft models utilised in papers II (MOLM-13) and III (OCI-AML3) benefit from being molecularly well characterised and of human origin. However, as illustrated by the bioluminescence imaging performed in paper II, cell line xenograft models don't always reflect a typical AML phenotype with leukemic burden not restricted to the bone marrow and spleen but spread out across various organs within the mice.

In paper III we utilised a PDX model to assess combination therapy using HU and VPA. Fluorescent monoclonal antibodies enabled imaging of engrafted mice showing the leukemic burden was predominantly restricted to the bone marrow in the spine and femurs. The PDX model has been well characterised and sequencing studies confirm that driver mutations, including FLT3-ITD and NPM1 mutation, that were present in the original patient sample are maintained after multiple passaging through NGS mice.

A significant limitation of PDX models, and preclinical modelling generally, is that any single model will fail to capture the molecular heterogeneity observed across diverse AML patients. To combat this, we explored a novel format of preclinical studies by testing multiple PDX models simultaneously in what we referred to as a preclinical trial. SDM was assessed simultaneously in 8 distinct PDX models each with distinct mutational profiles and disease latency. As illustrated in Figure 14A serdemetan treatment failed to significantly prolong the survival of xenografts as compared to untreated controls. However, in analysing the femurs of five of the PDX models available upon sacrifice indicated that the leukemic burden was substantially reduced in four of the models (Figure 14B).

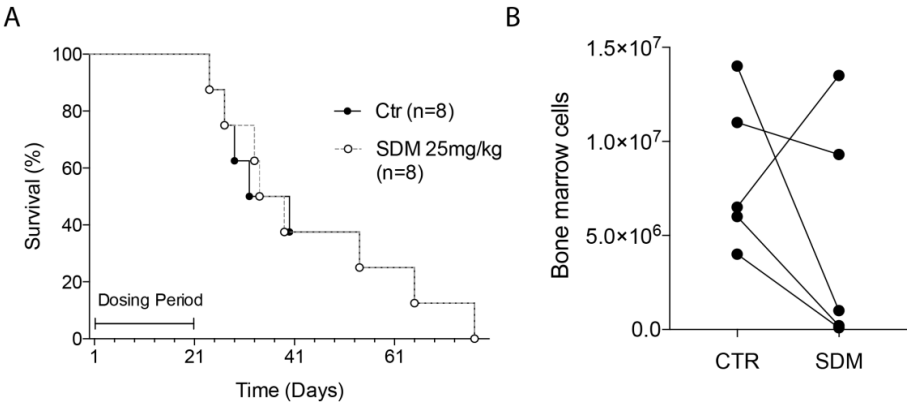

Figure 14. Preclinical trial model assessing SDM efficacy in 8 distinct AML PDX models. **(a)** Kaplan-Meier survival curve comparing 8 paired PDX samples, with variable disease latency and molecular characteristics, engrafted into 2 animals and sorted into two groups; untreated control (Ctr) and serdemetan treated daily for 21 days (SDM 25mg/kg). **(b)**

Comparison of the leukemic burden in the bone marrow (femurs) collected and processed from five of the models at time of sacrifice as determined by cell counting using standard light microscopy.

This pilot study demonstrates an interesting approach that could be developed further to enable preclinical screening of diverse AML patient types that could subsequently be investigated in more robust experimental settings.

A significant weakness associated with all the preclinical studies performed in papers II and III is the limited extent of biomarker or mechanism of action assays. In paper II additional animals could have been included and sacrificed immediately following cessation of treatment to compare Akt1 expression or autophagy markers in isolated leukemic cells from SDM treated animals and compared with control animals. Similarly, in paper III leukemic cells isolated from mice could be assayed for γH2AX expression to confirm the effect of combination induced DNS DSB was conserved *in vivo*.

6. Concluding remarks

The primary aim of the thesis was to contribute to the discovery and development of small molecules with therapeutic potential in AML.

In paper I we successfully supported the development of newly synthesised small molecules, Ag-NHC complexes, and characterised some of their intriguing cytotoxic properties in AML cell lines. In paper II we revaluated the industry developed agent, Serdemetan to determine its suitability for use in AML. SDM was performed well in preclinical animal models of AML and novel molecular targets including Akt and autophagy were uncovered. In paper III we repositioned two established anti-leukemic agents, hydroxyurea and valproic acid, as novel synergistic combination with potential for quick progression to clinical trials.

Through these distinct but related strategies for identifying and developing small molecules, the thesis successfully fulfilled its primary aim.

The identification of precise mechanisms of action or sensitive patient subgroups for each of the agents proved challenging and beyond this scope of the work, though intriguing clues were revealed that should guide future studies.

7. Future perspectives

The “omics” era of medicine continues to revolutionise molecular diagnostics and redefine the landscape of AML disease progression (188, 189). As such, small molecule development is inextricably bound to the concepts of personalised medicine and targeted therapy. Through reflection on the work performed in this thesis, particular concepts emerge as having special importance as to how small molecules, including those investigated in this thesis may be developed in the future.

Reimagining personalised therapy: It is possible and perhaps likely that the small molecules required to successfully treat most AML patients are already available. Based on this premise, a variety of integrated screening platforms have emerged in the previous decade (146, 190, 191). *Ex vivo* drug sensitivity and resistance testing (DSRT) enable primary tumour cells to be screened against vast libraries of compounds facilitating selection of personalised therapies. When integrated with omics data, such strategies provide a novel opportunity to therapeutically stratify heterogenous cancers, such as AML. In this context, the qualities of small molecules and their role in personalised medicine can be re-evaluated. Paper II attempts to characterise the tryptamine derivative, serdemetan and its therapeutic potential in AML. As a targeted therapy for specific inhibition of MDM2, SDM may be considered a failure, but this does not compromise its potential as a personalised therapy. SDM is a multitargeted agent capable of modulating a variety of pathways likely dependent on the molecular context of the exposed cells. SDM and other small molecules can therefore be seen to carry distinct disease-specific mechanistic profiles. For such compounds with convincing preclinical efficacy, it becomes reasonable to speculate that a subgroup sensitive of AML patients may be identified. Future development (or redevelopment) of small molecule therapies may emphasise identification of multitargeted agents with unique mechanistic profiles as characterised against the backdrop of AML disease heterogeneity.

Combination therapy and sequential regimens: Combination therapy for the treatment of AML attempts to increase efficacy and generate enduring therapeutic responses by

reducing acquired resistance. In paper III hydroxyurea (HU) was combined with valproic acid (VPA) and found to synergise with regard to their anti-leukemic activity. However, a more sophisticated understanding of how small molecules combine and functionally interact *in vivo* could enable novel scheduling regimens that greatly enhance therapeutic efficiency. Analysis of multiple cancer clinical trials with sufficient data to compare monotherapy and combination arms revealed that the majority of drug combinations are most likely operating with functional independence in treated patients (192). This observation has significant implications and explains why in some instances sequential administration of drugs may be as good or better than simultaneous combination therapy (193). Future studies performed with the small molecules developed in this thesis should be designed to guide optimal dosing schedules for clinical application. In the case of combining HU and VPA for the treatment of AML, a simple clinical trial could be designed to explore the benefit of combining the agents. HU is routinely administered to achieve leukocytoreduction in AML patients prior to induction of chemotherapy (194). A trial could be designed with the following two arms; (i) standardised HU therapy for cytoreduction (50 – 100 mg/kg per day) (ii) HU combined with a fixed dose of VPA. Short term clinical outcomes for such a study would focus on efficiency of cytoreduction as measured by white blood cell count, whilst long term analysis might investigate whether priming with HU and VPA combination treatment effects chemotherapy efficiency.

Small molecules as molecular tools: It is useful to imagine novel applications for small molecules in a broader vision of AML management. In Paper I alkylated imidazoles were complexed with silver (I) to generate novel compounds capable of inducing apoptosis in AML cell lines. It is hypothesised that the cytotoxicity of such metallodrugs is governed by their capacity to release metals from their auxiliary ligand into target cells. Cells treated with the compounds developed in Article I (NHC-1 and NHC-2) could be analysed by mass cytometry to directly measure silver uptake in exposed cells. Proof of concept studies were performed to confirm the validity of this approach during the PhD period though they were not included in the Paper I. Using this strategy preclinical studies would enable mapping and comparison of silver uptake in healthy versus leukemic tissues.

Placing novel small molecules into the AML therapeutic landscape: It is now clear that AML must be viewed as collection of distinct but related diseases. Furthermore, viewing an individual patient's disease progression across time reveals additional dimensions of therapeutic challenges and opportunities. Approximately half of all AML patients who achieve complete remission suffer relapse (195). Relapse disease is often mutationally distinct from diagnosis and associated with chemoresistance (196). Between remission and relapse, novel molecular technologies are revolutionising the ability to detect and characterise minimal residual disease (MRD (197). Novel small molecules should therefore be developed to target particular patient subgroups but also specific stages of disease progression. The combination of hydroxyurea and valproic acid explored in paper III may prove valuable as an alternative induction therapy for elderly or unfit patients. However, considering the mechanistic studies explored in our study, it may be expected that clones carrying mutated or dysfunctional p53 could emerge at relapse. Early detection of such clones during MRD or at relapse could encourage the use of an agent like serdemetan (paper II) as a consolidation therapy since it appears to target leukaemia cells independent of p53 status. Additionally, future small molecule development should be designed to support and compliment novel emerging therapeutic modalities. Immunotherapy, utilising biological drugs such as chimeric antigen receptor T cells or cell-based vaccines will almost certainly occupy a key position in the therapeutic landscape of AML (198, 199). To explore such therapies, we have developed a humanised preclinical mouse models of AML (200). Importantly, we have shown that a dendritic cell vaccination strategy can enhance and consolidate established small molecule therapy (venetoclax + 5-azacitidine) (201). To succeed, small molecules should be developed to compliment and harmonise with novel immunotherapies and generate sophisticated new therapeutic strategies for combating AML disease progression.

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Article I

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4-Alkylated Silver–N-Heterocyclic Carbene (NHC) Complexes with Cytotoxic Effects in Leukemia Cells

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Computational chemistry has shown that backbone-alkylated imidazoles ought to be efficient ligands for transition metal catalysts with improved carbene-to-metal donation. In this work, such alkylated imidazoles were synthesized and complexed with silver(I) by means of an eight/nine-step synthetic pathway we devised to access a new class of biologically active silver complexes. The synthesis involves selective iodination of the imidazole backbone, followed by Sonogashira coupling to replace the backbone iodine. The installed alkyne moiety is then subjected to reductive hydrogenation with Pearlman's catalyst. The imidazole N1 atom is arylated by the palladium-catalyzed Buchwald N-arylation method. The imidazole N3 position was then methylated with methyl iodine, whereupon the synthesis was terminated by complexation of the imidazolium salt with silver(I) oxide. The synthetic pathway provided an overall yield of ≈20%. The resulting complexes were tested in vitro against HL60 and MOLM-13 leukemic cells, two human-derived cell lines that model acute myeloid leukemia. The most active compounds exhibiting low IC_{50} values of 14 and 27 μM, against HL60 and MOLM-13 cells, respectively. The imidazole side chain was found to be essential for high cytotoxicity, as the imidazole complex bearing a C_7 side chain at the 4-position was four- to sixfold more potent than the corresponding imidazole elaborated with a methyl group.

Introduction

Numerous imidazoles are known for their potent biological activities,[1] including analgesic,[2] antibacterial,[3] cytotoxic,[4] and anticancer properties.[5] The imidazole framework is also an integral part of alkaloids,[6] and as a precursor for N-heterocyclic carbene (NHC) ligands[7] in organometallic catalysis.[8] Metallic silver has been known for centuries to possess bactericidal properties and has been used as a treatment for gonorrhea[9] before the development of modern antibiotics. In recent years, silver has reemerged as a viable option in the treatment of infectious diseases,[10] and silver-based products are currently used as topical antibacterial agents.[11] One such example is silver sulfadiazine,[12,13] which has been associated with delayed wound-healing.[14]

Silver–NHC complexes[15,16] have been found to possess both antibiotic[17] and anticancer properties[18] and have an untapped potential as drug candidates. Furthermore, studies have shown that synergistic effects that involve both the silver and the NHC ligand play a profound role in the cytotoxicity of such complexes.[19]

Theoretical calculations performed at our laboratories revealed that substitution on the imidazole backbone is beneficial for ligand-to-metal donation.[20] We envisioned that imidazoles elaborated with aliphatic groups on the backbone would have a less labile C–Ag bond and thus be able to afford slow release of silver, an effect that we believe could be beneficial for a potential metallodrug. Although a great number of imidazole-based silver complexes have been reported, only few bear backbone substitution such as bis-methylation,[21a] bis-chlorination,[21b] and alkenylation[21c] (Figure 1). However, the synthesis and biological activity of imidazoles elaborated with aliphatic alkyl chains on the backbone have not been previously reported. To date, such substitution patterns have been difficult to approach, due to lack of synthetic methodology. In fact, Ag–NHC complexes have been realized by elaboration of imidazoles with embedded functionality,[21] or classical condensation reactions from linear precursors.[22] A major drawback to these strategies is low synthetic flexibility and inferior structural diversity achieved in the target imidazoles.

To address these challenges, we have devised and developed new synthetic methods for the preparation of 4-alkylated imidazole–silver complexes. Along with this work, we have revealed new synthetic methods for imidazole functionalization that include selective halogenation,[23] Suzuki cross-coupling,[24]

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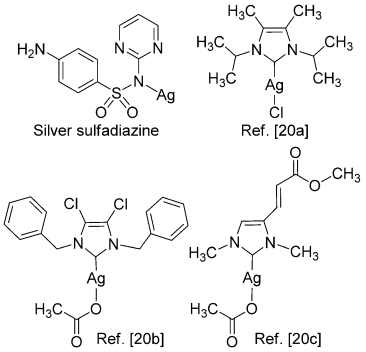


Figure 1. Previously disclosed biological active organosilver complexes.

Stille coupling,[25] and a method for Sonogashira coupling,[26] the latter of which was used in the study described herein.

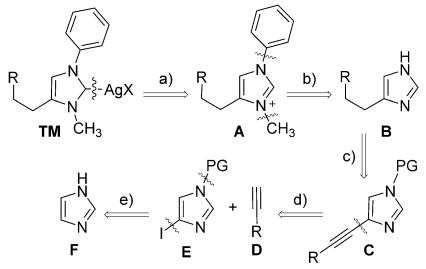
Results and Discussion

Chemistry

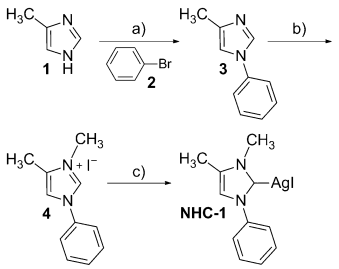
A retrosynthetic analysis to our target Ag–NHC is outlined in Scheme 1. We envisioned that the target (**TM**) could be produced by complexation of imidazolium salt **A** with a suitable silver salt. Two regioselective N-substitution reactions from 4-(5)-alkylated-1*H*-imidazole **B** might lead to the desired salt **A**. The backbone-substituted imidazole **C** could be produced from the iodoimidazole **E** and an appropriate alkyne **D** through a Sonogashira coupling reaction. The key intermediate **E** could be produced from commercially available imidazole **F**.

To directly compare the effect on the activity of the complexes with increasing length of the side chains, we devised two different target molecules, **NHC-1** (Scheme 2) and **NHC-2** (Scheme 3), which differ by one feature, namely the substituent at the imidazole backbone position 4; **NHC-1** contains a methyl group, and **NHC-2** contains a heptyl group. The two NHC–silver complexes of this study can be prepared via two different pathways. Because 4-methylimidazole **1** is commercially available, a short pathway leading to **NHC-1** was established, a synthesis that comprises a) N-arylation,[27] b) N-methylation,[28] and c) complexation with silver[29] to obtain the **NHC-1** silver complex. The overall sequence provides a yield of 30%, which corresponds to a mean step yield of 67%.

The devised synthesis leading to **NHC-2** (Scheme 3) commenced with the preparation of *N*-toluenesulfonyl-4-iodoimidazole **4**, which served as a key intermediate for the synthesis of backbone-alkylated imidazoles. The synthetic pathway **1**→**4** involves a di-iodination step (a) followed by selective de-iodination (b), or a selective mono-iodination step (a′). The 4-iodinated imidazole is then subjected to the introduction of an auxiliary group at the N-tosylation step (c).[30] With the key substrate **4** in hand, we used our recently disclosed method for Sonogashira coupling[26] to perform the desired C–C coupling reaction of step (d). Reduction of the alkyne bond of **10** was



Scheme 1. Retrosynthetic analysis leading to 4-substituted imidazolium silver complexes **TM**.

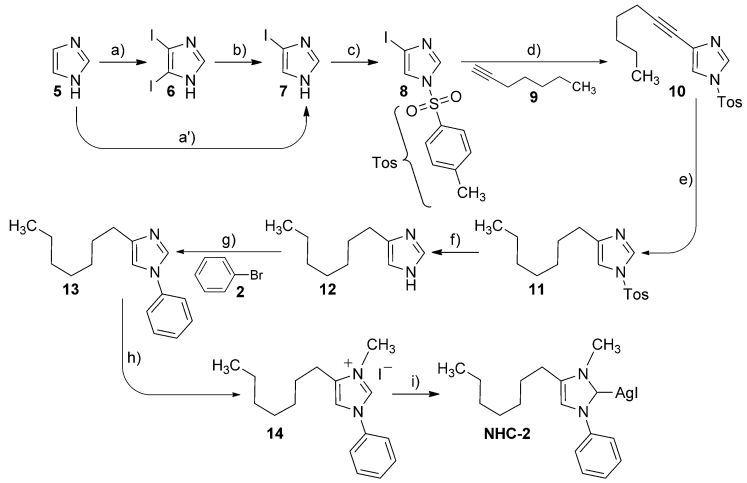


Scheme 2. Synthesis of *N*-1-phenyl-*N*-3-methyl-4-methylimidazol-2-yliden silver iodide **NHC-1**. *Reagents and conditions*: a) PhBr (**2**), $Pd_2(dba)_3$, $Me_4tBuXPhos$, K_2HPO_4, toluene, 120 °C, 5 h, 87%; b) MeI, THF, reflux, 4 h, 62%; c) Ag_2O, CH_2Cl_2, RT, 3 h, 56%.

performed in excellent yield (92%) by means of Pearlman's catalyst ($Pd(OH)_2/C$) in methanol under a hydrogen atmosphere. The following step, removal of the toluenesulfonyl auxiliary group, was performed by treatment with concentrated hydrochloric acid in methanol at reflux in excellent yield (98%). The liberated imidazole derivative **12** was N-arylated [step (g)] by using bromobenzene in toluene with $Pd_2(dba)_3$ and $Me_4tBuXPhos$ as ligand[27] in good yield (78%). The last organic reaction step of the synthetic pathway involved conversion of the imidazole derivative into an imidazolium salt[28] **14** from the *N*-aryl-4-subsituted imidazole derivative **13** by reacting with methyl iodide at reflux in THF to obtain the *N*1-phenyl-*N*3-methyl-4-methylimidazolium iodine salt **14** (57%). Finally, the target silver salts were prepared by using silver(I) oxide in dichloromethane.[29] The nine-step synthesis providing **NHC-2** afforded an overall yield of 19%, which corresponds to a mean step yield of 81%.

Biology

The cytotoxic potential of metallodrugs may be influenced by their capacity to release metals from the associated auxiliary ligand. We hypothesized that the varied nature of the R group of the 4-substituted imidazoles may further impact the cytotoxic potential of the compounds. To compare the biological



Scheme 3. Synthesis of *N*-1-phenyl-*N*-3-methyl-4-heptylimidazol-2-yliden silver iodide **NHC-2**. *Reagents and conditions*: a′) DIH, H_2SO_4 (cat.), H_2O, 0 °C, 81%; a) I_2, KI, NaOH, RT, 24 h, b) $Pd(OAc)_2$ (0.15%), XPhos, K_2HPO_4, MeOH, H_2O, reflux, 90 min, (quant.); c) TosCl, NEt_3, THF, RT, 24 h, 68%; d) $Pd(PPh_3)_4$, CuI, NEt_3, DMF, MW, 80 °C, 30 min, 79%; e) H_2 (1 atm), $Pd(OH)_2$/C, MeOH, RT, 24 h, 92%; f) HCl, MeOH, reflux, 2 h, 98%; g) PhBr (**2**), $Pd_2(dba)_3$, Me_4*t*BuXPhos, K_2HPO_4, toluene, 120 °C, 5 h, 78%; h) MeI, THF, reflux, 4 h, 57%; i) Ag_2O, CH_2Cl_2, RT, 3 h, 90%.

activity of the compounds, we incubated **NHC-1** and **NHC-2** with the human acute myeloid leukemia cell lines HL60 and MOLM-13. Following 24 h incubation, the WST1 viability assay was performed, revealing the estimated IC_{50} values of the compounds to vary as a function of both the side chain R group and the cell type used (Figure 2). The p53-null cell line, HL60, proved more sensitive to both compounds. **NHC-1** showed an IC_{50} value of 78 μM in HL60 cells, compared with an IC_{50} value of 123 μM in MOLM-13 cells. A similar trend was observed for the more potent **NHC-2** (HL60 IC_{50}: 14 μM; MOLM-13 IC_{50}: 27 μM). To confirm the compounds are truly cytotoxic and not only antiproliferative, we performed nuclear staining with Hoechst 33342 in both cell lines after 24 h incubation (**NHC-1** at 100 μM, **NHC-2** at 30 μM). Condensed and fragmented nuclei were observed in both cell lines and are characteristic of apoptosis (Figure 2).

Using the WST1 assay to compare cell viability at 4, 12, and 24 h, we discovered the compounds (**NHC-1** at 100 μM and **NHC-2** at 20 μM) to induce rapid cell death. For both cell lines and complexes the majority of cell death was observed after 4 or 12 h (Figure 3). The rapidity of death was best exemplified in the HL60 cell line. Finally, we performed flow cytometry combined with Annexin V staining in HL60 cells treated with **NHC-1**, **NHC-2**, or the chemotherapeutic pyrimidine analogue cytarabine (arabinofuranosyl cytidine) for comparison. Cell viability was determined by forward and side scatter properties (Figure 4). Exposure of the lipid membrane component phosphatidylserine (PS) allows its staining with Annexin V; this is indicative of apoptosis. Furthermore, PS exposure aids to effectively eliminate large numbers of dying cells from the systemic circulation without releasing noxious intracellular material.[31] Significant PS staining was observed exclusively in HL60 cells treated with **NHC-1** and **NHC-2**, but was absent in cytarabine-treated cells. Our experiments confirm the cytotoxic potential of the silver–imidazole complexes and suggest that cell death in HL60 cells is mechanistically distinct from that which occurs in cells treated with the chemotherapeutic cytarabine.

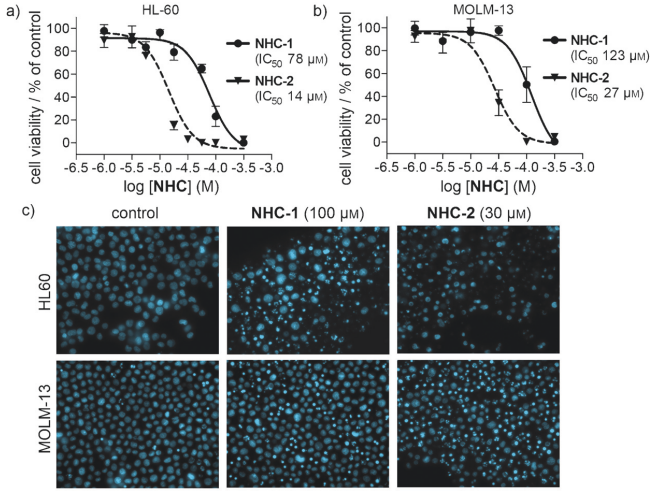


Figure 2. NHC-1 and **NHC-2** are cytotoxic in leukemia cell lines. a) HL60 and b) MOLM-13 cells were treated with **NHC-1** and **NHC-2** for 24 h, and cell viability was determined by WST1 assay to generate dose–response curves. Experiments were performed in three independent replicates, and data are the mean ± SD. c) HL60 and MOLM-13 cells were treated with **NHC-1** (100 μM) and **NHC-2** (30 μM) as indicated for 24 h, and nuclear morphology was determined by Hoechst 33342 staining to identify dead cells.

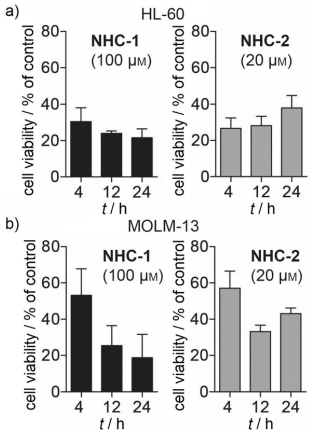


Figure 3. NHC-1 and **NHC-2** induce rapid cell death in leukemia cell lines. a) HL60 cells (TP53-null, FLT3 wild-type) were treated with **NHC-1** (100 μM) and **NHC-2** (20 μM) for 4, 12, and 24 h, and viability was determined by WST1 cell viability assay. b) MOLM-13 cells (TP53 wild-type, FLT3-internal tandem mutation) were treated with **NHC-1** (100 μM) and **NHC-2** (20 μM) for 4, 12, and 24 h, and viability was determined by WST1 cell viability assay. Experiments were performed in three independent replicates, and data are the mean ± SD.

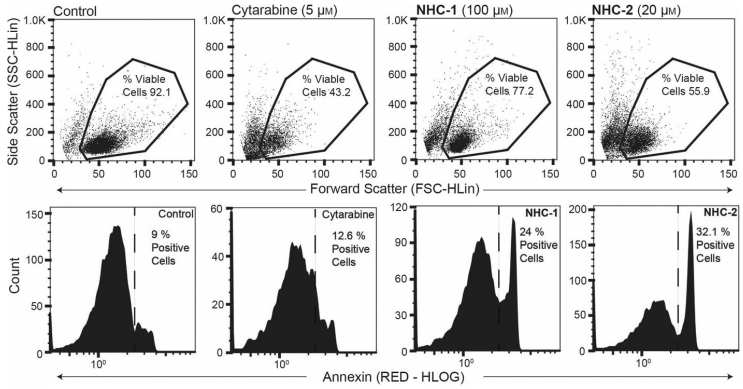


Figure 4. NHC-1 and **NHC-2** induce expression of cell-surface phosphatidylserine (PS) in HL60 cells. Cells were treated with **NHC-1** (100 μM), **NHC-2** (20 μM), or cytarabine (5 μM) for 24 h. Cell viability was determined by comparing the forward scatter and side scatter properties with those of untreated control cells using flow cytometry, as illustrated by figures in the upper row. Gated events represent viable cells. PS expression was determined by Annexin V staining and analysis by flow cytometry as illustrated by histograms in the lower row. Events to the right of the dashed lines are considered positive for Annexin V as determined by comparison with unstained control cells. All analyzed cells are taken from events deemed viable.

Conclusions

A novel class of Ag–NHC complexes encompassing imidazoles furnished with alkyl side chains on the backbone 4-position have been realized by de novo synthesis using new methods developed in our research group. Two Ag–NHC complexes were prepared with different side chains: **NHC-1** (methyl) and **NHC-2** (heptyl). Both complexes were found to be potently cytotoxic against two human leukemia cell lines, HL60 and MOLM-13, in the micromolar range. IC_{50} values for **NHC-2** were 14 and 27 μM, respectively, and are motivation for further development as an anticancer therapy. As the demand for novel alternative cancer therapeutics remains unmet, metallodrugs are an increasingly important compound class for investigation. The unique anti-leukemic properties of the Ag–NHC complexes described herein further underscore the value of exploring novel synthetic organometallic chemistry in drug development.

Experimental Section

Chemistry

GC analyses were performed on a capillary gas chromatograph equipped with a fused silica column (*l*: 25 m, i.d.: 0.20 mm, film thickness: 0.33 μm) at a helium pressure of 200 kPa, split less/split injector and flame ionization detector. DART-MS spectra were obtained using PEG as an internal standard under positive ionization mode with a ToF mass analyzer. ^{1}H and ^{13}C NMR spectra were recorded on instruments operating at 400 and 150 MHz, respectively. Chemical shifts were referenced to the deuterated solvent used in that experiment. All melting points are uncorrected. Synthesis of precursors **2**, **3**, **4**, and **10** were reported previously. The microwave-assisted experiments were performed with a Biotage Initiator Sixty EXP Microwave System operating at 0–400 W at 2.45 GHz. The instrument operates in the temperature range 40–250 °C, a pressure interval of 0–20 bar (2 MPa, 290 psi) with reactor vial volumes of 0.2–20 mL. Multiple attempts to grow crystals of **NHC-1** and **NHC-2** suitable for X-ray analysis were performed in various solvent systems, but unfortunately only amorphous material was obtained in most cases.

***N*-Toluenesulfonyl-4-heptylimidazole 11**. Imidazole (0.10 g) and $Pd(OH)_2$/C (15% *w*/*w*) were transferred to a round-bottom flask (50 mL) equipped with a magnetic stir bar. MeOH (25 mL) was added, and the flask was evacuated under reduced pressure and flushed with H_2 from a balloon three times. The reaction mixture was stirred vigorously at room temperature for 24 h. The post-reaction mixture was filtered through a pad of Celite that was subsequently washed with multiple small portions of MeOH. The solvent was evaporated to give the product as a tan oil in 92% yield (purity: ≥95% as determined by GC) without need for further purification. ^{1}H NMR ($CDCl_3$): $\delta = 7.91$ (s, 1H), 7.79 (d, 2H, $J = 8.0$ Hz), 7.34 (d, 2H, $J = 7.0$ Hz), 2.48 (t, 2H, $J = 7.4$ Hz), 1.57 (2H, m), 1.23 (8H, m), 0.86 ppm (t, 3H, $J = 7.1$ Hz); ^{13}C NMR ($CDCl_3$): $\delta = 146.1$, 136.3, 135.4, 130.5, 127.4, 112.9, 31.9, 29.3, 29.1, 28.7, 28.3, 22.7, 21.8, 14.2 ppm; HRMS (DART): *m*/*z* $[M+H]^+$ calcd for $C_{17}H_{25}N_2O_2S$: 321.16367, found: 321.16367.

4-(5)-Heptyl-1*H*-imidazole 12. Imidazole (2.94 mmol, 0.20 g) was dissolved in MeOH (20 mL) in a round-bottom flask (25 mL). HCl (concd, 1 mL) was added to this mixture in one portion. The reaction mixture was held at reflux for 2 h, whereupon the MeOH was evaporated and HCl (3 M, 10 mL) was added. The resulting mixture was extracted with Et_2O (2×20 mL), and the organic phases discarded. The aqueous phase was made alkaline with NaOH (4 M) and again extracted with Et_2O (3×20 mL). The organic extracts were combined, dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure to provide the product at high purity. The product was isolated as a tan oil in 98% yield. ^{1}H NMR ($CDCl_3$): δ = 11.07 (1H, m, br), 7.64 (1H, s), 6.76 (1H, s), 2.59 (t, 2H), 1.61 (m, 2H), 1.26 (m, 8H), 0.85 ppm (t, 3H); ^{13}C NMR ($CDCl_3$): δ = 137.0, 134.2, 117.7, 31.9, 29.4, 29.4, 29.2, 26.6, 22.8, 14.2 ppm; HRMS (DART): *m/z* $[M+H]^+$ calcd for $C_{10}H_{19}N_2$: 167.15482, found: 167.15466.

General procedure for Pd-catalyzed N-arylation coupling reaction between 4-(5)-alkyl-1*H*-imidazoles and bromobenzene.[27] To an oven-dried tube was transferred imidazole (1.2 mmol), K_3PO_4 (424 mg, 2.0 mmol), and bromobenzene (2.0 mmol). The vial was sealed with a septum and carefully flushed with argon through the septum. A second oven-dried tube was charged with $Pd_2(dba)_3$ (0.0125 mmol) and Me$_4$*t*BuXPhos (0.025 mmol) before it was sealed with a septum and flushed with argon. The catalyst was dissolved in a mixture of anhydrous toluene (0.83 mL) and anhydrous 1,4-dioxane (0.17 mL), and the resulting dark-purple mixture was stirred at 120 °C for 3 min, at which point the color of the mixture turned to red–brown. The catalyst was then transferred to the first vial, and the reaction mixture was heated at 120 °C for 5 h. At the end of the reaction time, the mixture was cooled to room temperature, diluted with EtOAc (10 mL), washed with brine (2 mL), and dried over $MgSO_4$. The drying agent was filtered off, and the organic solvent was removed under reduced pressure. The crude product was then purified by flash chromatography with the eluent systems specified below.

***N*-Phenyl-4-methylimidazole 3**. Isolated as a yellow oil by silica gel column chromatography [EtOAc/hexanes (4:6)→(1:1)] in 87% yield. ^{1}H NMR ($CDCl_3$): δ = 7.74 (1H, s), 7.44 (2H, t, *J* = 7.6 Hz), 7.33 (3H, m), 6.99 (s, 1H), 2.29 ppm (s, 3H); ^{13}C NMR ($CDCl_3$): δ = 139.6, 134.6, 134.7, 129.9, 127.1, 121.1, 114.7, 13.8 ppm.

***N*-Phenyl-4-heptylimidazole 13**. The product was isolated by flash silica gel chromatography [EtOAc/hexanes (1:9)→(1:1)] as pale crystals in 78% yield. R_f = 0.58 [EtOAc/hexanes (1:1)]; mp: 53.9–55.0 °C; ^{1}H NMR ($CDCl_3$): δ = 7.77 (s, 1H), 7.45 (t, 2H, *J* = 7.5 Hz), 7.33 (m, 3H, *J* = 8.3 Hz), 7.00 (s, 1H), 2.62 (t, 2H, *J* = 7.8 Hz), 1.69 (t, 2H, *J* = 7.4 Hz), 1.31 (m, 8H), 0.87 ppm (t, 3H, *J* = 7.4 Hz); ^{13}C NMR ($CDCl_3$): δ = 144.8, 137.7, 134.7, 129.9, 127.2, 121.3, 114.2, 320, 29.5, 29.5, 29.5, 29.3, 28.6, 22.8, 14.3 ppm; HRMS (DART): *m/z* $[M+H]^+$ calcd for $C_{16}H_{23}N_2$: 243.18558, found: 243.1860.

General procedure for N-3-methylation of N-1-phenyl-4-alkylimidazole.[28] *N*-phenyl-4-alkylimidazole (2.0 mmol) was dissolved in THF (15 mL). Methyl iodide (4.00 mmol) was added in one portion, and the reaction mixture was held at reflux for 4 h. The post-reaction mixture was allowed to cool, resulting in crystallization of the product which was filtered, washed with small portions of hexane, and air-dried to furnish the product.

***N*-1-Phenyl-*N*-3-methyl-4-methylimidazolium iodide**. Isolated as a white solid in 62% yield. ^{1}H NMR ($CDCl_3$): δ = 10.12 (s, 1H), 7.65 (d, 2H, *J* = 7.8 Hz), 7.60 (s, 1H), 7.42 (m, 3H), 3.98 (s, 3H), 2.37 ppm (s, 3H); ^{13}C NMR ($CDCl_3$): δ = 135.2, 134.5, 132.8, 130.7, 130.3, 122.0, 117.8, 34.9, 9.7 ppm; HRMS (ESI): *m/z* $[M]^+$ calcd for $C_{11}H_{13}N_2$: 173.10787, found: 173.10779; $[M_2I]^+$ calcd for $C_{22}H_{26}N_4I$: 473.12021, found: 473.12594.

***N*-1-Phenyl-*N*-3-methyl-4-heptylimidazolium iodide**. Isolated as a tan solid in 57% yield. ^{1}H NMR ($CDCl_3$): δ = 10.60 (s, 1H), 7.74 (d, 2H, *J* = 7.9 Hz), 7.54 (m, 3H), 4.12 (s, 3H), 2.69 (t, 2H, *J* = 8.0 Hz), 1.71 (m, 3H), 1.35 (m, 7H), 0.87 ppm (t, 3H, *J* = 6.9 Hz); ^{13}C NMR ($CDCl_3$): δ = 137.3, 135.9, 134.7, 130.8, 130.4, 122.1, 116.7, 34.9, 31.7, 29.3, 29.0, 27.3, 23.9, 22.8, 14.2 ppm; HRMS (DART): *m/z* $[M+H-I]^+$ calcd for $C_{17}H_{25}N_2$: 257.20177, found: 257.20179.

General procedure for the synthesis of imidazole-based silver complexes.[29] Imidazolium salt (1.77 mmol) was dissolved in CH_2Cl_2 (15 mL), and silver(I) oxide (0.89 mmol) was added in one portion. The black reaction mixture was stirred for 2 h 25 min, at which point the mixture became pale brown. The solution was poured into a beaker containing hexanes (100 mL), resulting in precipitation of a crystalline solid. This was filtered and recrystallized from CH_2Cl_2 to furnish the silver complexes.

***N*-1-Phenyl-*N*-3-methyl-4-methylimidazol-2-yliden silver(I) iodide**. Isolated as a white solid in 56% yield after pooling three crops. ^{1}H NMR ($CDCl_3$): δ = 7.52 (m, 2H), 7.39 (m, 3H), 6.98 (s, 1H), 3.82 (s, 3H), 2.29 ppm (s, 3H); ^{13}C NMR ($CDCl_3$): δ = 140.4, 131.3, 129.8, 1286, 124.1, 119.0, 36.9, 10.0 ppm; HRMS (DART): *m/z* $[M+H-AgI]^+$ calcd for $C_{11}H_{13}N_2$: 173.10732, found: 173.10215.

***N*-1-Phenyl-*N*-3-methyl-4-heptylimidazol-2-yliden silver(I) iodide**. Isolated as a white solid in 90% yield. ^{1}H NMR ($CDCl_3$): 7.54 (d, 2H), 7.42 (m, 3H), 6.95 (s, 1H), 3.84 (s, 3H), 2.58 (m, 2H), 1.65 (m, 2H), 1.30 (m, 8H), 0.89 ppm (t, 3H); ^{13}C NMR ($CDCl_3$): δ = 182.6, 140.4, 136.0, 129.9, 128.7, 124.0, 118.1, 36.7, 31.8, 29.3, 29.1, 27.8, 24.5, 22.8, 14.2 ppm; HRMS (DART): *m/z* $[M-AgI]^+$ calcd for $C_{17}H_{25}N_2$: 257.20177, found: 257.20156.

Biology

The human cell lines MOLM-13 and HL60 were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in RPMI 1640 (Invitrogen), containing 10% heat-inactivated fetal bovine serum (GE Healthcare, Life Sciences), 2 mM L-glutamine, and 50 U mL^{-1} penicillin/streptomycin (Sigma–Aldrich). Evaluation of viability/apoptosis was performed as described previously.[32, 33] Cell analysis after drug treatment (2×10^5 cells per mL) was carried out by fixing cells in 8% formaldehyde in PBS, DNA-specific staining with Hoechst 33342 (Invitrogen; 10 μg mL^{-1}), followed by counting of normal and fragmented/condensed cell nuclei in an inverse fluorescence microscope (Zeiss Axio Vert.A1), or by flow cytometric analysis and Annexin staining. Annexin staining (Invitrogen) was performed in accordance with the manufacturer's recommended procedure and run on the Guava easyCyte flow Cytometer (EMD Millipore). The WST1 assay cell proliferation reagent (Life Sciences) was used in accordance with the manufacturer's procedure, followed by respective reading of luminescence and absorbance (Spectra Max Gemini EM, Molecular Devices). All cell viability assays were performed in flat-bottomed 96- or 24-well tissue culture test plates.

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Keywords: cytotoxicity · imidazoles · leukemia · metallodrugs · silver

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Article II

Article III

Hydroxyurea synergizes with valproic acid in wild-type p53 acute myeloid leukaemia

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ABSTRACT

Palliative care in acute myeloid leukaemia (AML) is inadequate. For elderly patients, unfit for intensive chemotherapy, median survival is 2–3 months. As such, there is urgent demand for low-toxic palliative alternatives. We have repositioned two commonly administered anti-leukaemia drugs, valproic acid (VPA) and hydroxyurea (HU), as a combination therapy in AML.

The anti-leukemic effect of VPA and HU was assessed in multiple AML cell lines confirming the superior anti-leukemic effect of combination therapy. Mechanistic studies revealed that VPA amplified the ability of HU to slow S-phase progression and this correlated with significantly increased DNA damage. VPA was also shown to reduce expression of the DNA repair protein, Rad51. Interestingly, the tumour suppressor protein p53 was revealed to mitigate cell cycle recovery following combination induced arrest. The efficacy of combination therapy was validated *in vivo*. Combination treatment increased survival in OCI-AML3 and patient-derived xenograft mouse models of AML. Therapy response was confirmed by optical imaging with multiplexed near-infrared labelled antibodies.

The combination of HU and VPA indicates significant potential in preclinical models of AML. Both compounds are widely available and well tolerated. We believe that repositioning this combination could significantly enhance the palliative care of patients unsuited to intensive chemotherapy.

INTRODUCTION

Palliative care in acute myeloid leukaemia (AML) is unsatisfactory. For elderly patients, who do not tolerate intensive chemotherapy or bone marrow transplantation, median survival is 2–3 months [1, 2]. AML is a genetically heterogeneous disease and numerous classes of anticancer agents have been trialled with varying success. Histone deacetylase inhibitors (HDACi) have indicated significant potential, whilst DNA targeting compounds remain a stalwart of clinical practice [3]. Emerging studies continue to reveal novel molecular mechanisms underpinning these agents. Such molecular insights also provide a unique opportunity to reassess existing therapeutics.

Valproic acid (VPA) is a short chain fatty acid used clinically as an anticonvulsant for more than 30 years. In 2001 the compound was rediscovered for its anticancer activity as an HDAC inhibitor, (HDACi) targeting class 1 and 2 HDAC enzymes [4]. A multitude of preclinical studies have combined VPA with genotoxic and non-genotoxic therapies reporting substantially increased efficacy [5–8]. More recently it has been illustrated that this increased efficacy may be accountable to VPA's capacity to modulate DNA damage repair proteins. Specifically VPA has been shown to target homologous recombination proteins, negatively regulating expression and localization of Rad51, Chk1, BRCA1 and BRCA2. [9] In the last decade various clinical trials have exhibited

VPA's capacity to incite clinical response in primary and secondary AML patients [10–13]. However, the use of VPA in AML is predicated on its HDACi activity and consequent induction of differentiation and apoptosis. Limited clinical emphasis has been placed on its ability to modulate DNA repair.

Hydroxyurea (HU) is an antimetabolite that targets cancer cells through stalling and subsequent collapse of S-phase replication forks [14]. HU inhibits ribonucelotide reductase thereby depleting the cellular pool of deoxynucleotides and incurring reversible DNA damage [15]. S-phase specific genomic assaults then depend on homologous recombination proteins, including Rad51, for repair [16]. The clinical importance of HU in myeloid neoplasms cannot be understated. Hydroxyurea is routinely administered to achieve leukocytoreduction in AML, chronic myeloid leukaemia, hyperleukocytosis and leukostasis [17, 18]. The safety of the compound is further exemplified by its on-going use and reccomendation for the treatment of young patients suffering from sickle cell anaemia [19]. In AML the compound may be prescribed prior to induction chemotherapy or experimentally in combination with other moderate or low toxic therapies, particularly in palliative care [20, 21].

Various preclinical studies have examined the synergistic effects of combining genotoxic compounds and HDACi [22–25]. However, the mechanisms described to account for the observed synergism remain inconclusive and may differ substantially depending on the models explored. Based on our preclinical and clinical experience with VPA we are appreciating its relevance in palliative care of AML [5, 26, 27]. Furthermore non-systematic testing of VPA and HU in single patients suggests the combination is well tolerated in elderly patients [21]. These factors encouraged us to reassess HU and VPA as a combination in preclinical models of AML. Here we provide evidence that HU and VPA combine effectively in AML through cooperative modulation of the cell cycle and DNA repair proteins. Additionally we identify intact tumour suppressor protein p53 as a likely predictor of therapeutic response to the combination. *In vivo* imaging and survival analysis in orthotopic mouse models, including a patient-derived xenograft model, confirmed that this combination treatment improves survival. The established tolerance and low toxicity of these compounds additionally highlights their potential in the palliative care of elderly AML patients.

RESULTS

HU and VPA cooperatively induce cell death in p53 wild-type leukaemia cell lines

The cell death capacity of HU and VPA alone and in combination was assessed in four AML cell lines (MV4–11, OCI-AML3, MOLM-13, and HL-60) using Hoechst 33342 nuclear staining. Cells were treated at a fixed ratio alone or in combination for 72 hours with increasing doses of HU (25–200 μM) and VPA (0.25–2 mM) (Figure 1A–1D). Combination treatment consistently enhanced cell death induction as compared to the single agents in all cell lines. However, when comparing the cell viability at doses (HU 50 μM and VPA 0.5 mM) best reflecting patient serum concentrations [10, 21], the p53 null HL-60 cells were identified as the most resistant cell line (Figures 1A–1D). To examine whether p53 status can mediate therapy sensitivity at clinically relevant doses, 3 additional leukemic cell lines (KG1-A, THP-1 and K562) harbouring p53 mutations were assessed and compared to the cell lines previously described. All cell lines were exposed to HU 60 μM and VPA 0.6 mM for 72 hours to reflect clinically achievable concentrations [10]. Cell death in response to combination therapy was significantly increased in wild-type p53 cell lines compared to null or mutated p53 cell lines. Comparatively, single agent therapy failed to distinguish significantly between cell lines with varying p53 status (Figures 1E–1G). To further investigate the significance of p53 status in response to HU and VPA combination therapy, we employed MOLM-13 cells expressing shRNA targeting p53 gene expression. Western blotting confirmed reduced expression of the p53 protein in MOLM-13 shp53 cells when compared with MOLM-13 wt p53 cells transduced with an untargeted empty vector (Figure 2A) The two cell lines were treated with HU (75 μM and 100 μM), VPA (0.75 mM and 1 mM) or the combinations. Cell death was determined by flow cytometry using Annexin-PI staining following 72 hrs treatment (Figure 2B–2C). At both concentration ratios, the combination therapy induced significantly more death in MOLM-13 wt p53 cells when compared with MOLM-13 shp53 cells. It is a growing concern that chemotherapy may select for a minority of p53 mutant clones in AML patients [28]. This may contribute significantly to the emergence of therapy resistant relapse disease. To investigate the enduring effect of the combination therapy, cells were exposed to HU (100 μM), VPA (1 mM) and the combination for 72 hrs. Cells were then washed twice and reseeded in drug free medium and maintained for a further 72 hrs. Viable cells were counted at 24 hr intervals throughout the course of the experiment (6 days). This recovery assay was performed in MOLM-13 shp53, MOLM-13 wt p53 (Figure 2D–2G), HL-60 ($p53^{null}$) and OCI-AML3 ($p53^{wild\text{-}type}$) cells (Figures H-K). In all cell lines untreated control cells displayed typical growth curves over the 6 day period, whilst VPA exerted a mild slowing of division rate that was lost with removal of the treatment. HU exhibited a more profound arrest in cell division, particularly in cells with wild-type p53 status. However, again all cell lines were able to recover upon removal of the treatment. Uniquely, the combination therapy limited recovery to the HL-60 and MOLM-13

shp53 cell lines, with treatment resulting in a terminal arrest of MOLM-13 wtp53 and OCI-AML3 cells. The presence of substantial p53 expression therefore appears crucial to induction of a lasting anti-leukemic effect with this combination.

HU and VPA cooperatively regulate cell cycle in OCI-AML3

Given the apparent significance of the role of p53 in combination treatment response, OCI-AML3 (p53

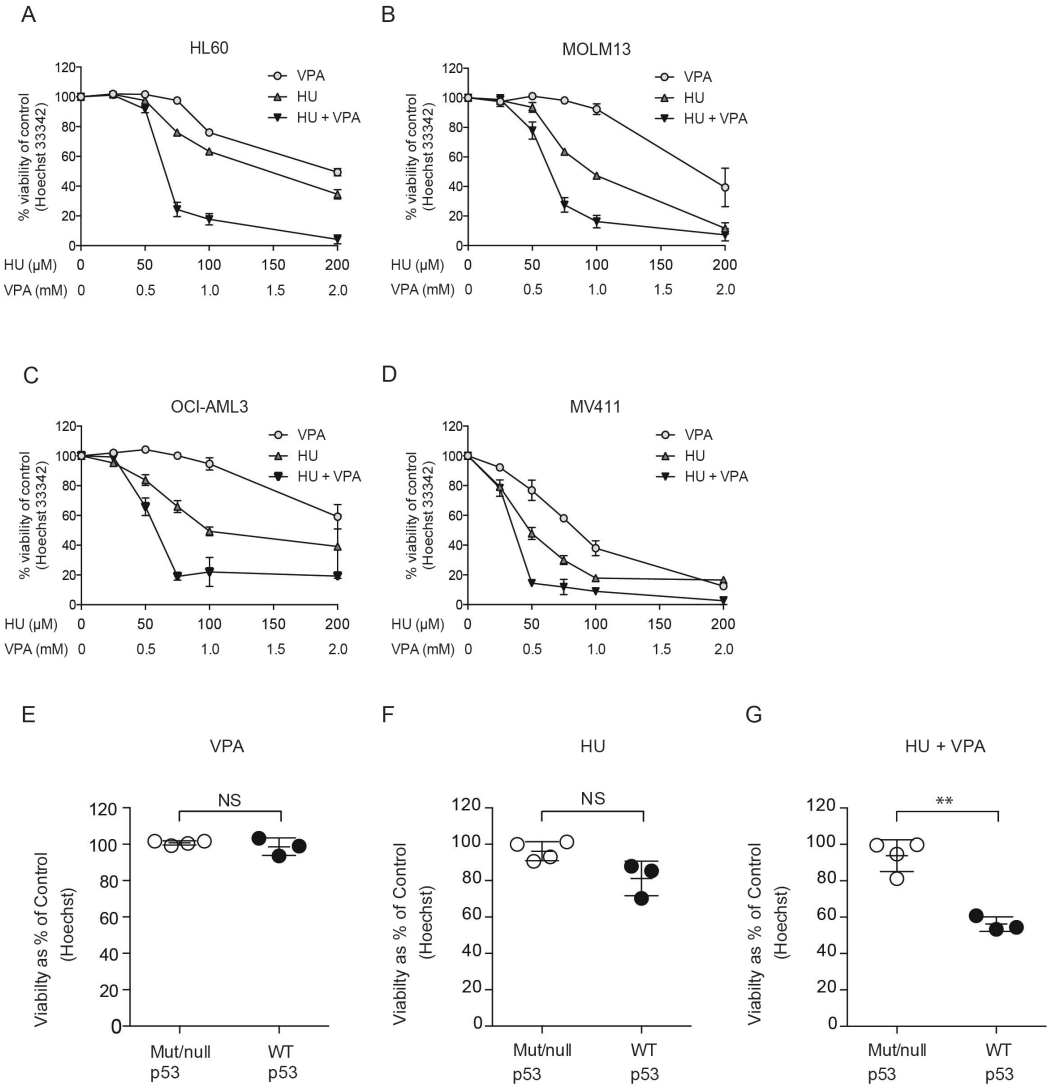

Figure 1: Assessment of cell death induction and the enhanced potential of combining HU and VPA in AML cell lines. (**A-D**) Hoechst nuclear staining assay performed to generate dose response curves for cell death induction. HL-60, MOLM-13, OCI-AML3 and MV4-11 cells were treated with HU (25–200 μM) and VPA (0.25–2 mM) alone or in combination at a fixed ratio (1:10) for 72 hrs. $N = 3$. (**E-G**) Hoechst nuclear staining assay is performed to determine the % of viable cells in MOLM-13, OCI-AML3, MV4-11 (p53 wild-type), KG1-A, THP-1, K562 (p53 mutated) and HL-60 cell lines. All cell lines were treated with HU (60 μM), VPA (0.6 mM) and the combination. Results are pooled and presented as mutated/null p53 (Mut/null p53) vs. wild-type p53 cell lines (WT p53). A significant increase in sensitivity was observed in WT p53 cell lines under combination treatment (**$P < 0.01$) $N = 3$.

wild-type) cells were selected for mechanism of action studies. Previous studies have determined that the combination of HU and VPA can cooperatively arrest cell cycle progression resulting in enhanced cell death [23]. We assessed the cell cycle status of OCI-AML3 cells following 24 hrs exposure to HU (100 μM) and VPA (1 mM), both alone and in combination (Figure 3A). HU slowed cell cycle progression with OCI-AML3 cells accumulating in S and G2/M phase. VPA´s capacity to induce G1 arrest is previously described [29], however OCI-AML3 cells were only mildly arrested following VPA exposure for 24 hrs (Control 51.7% vs. VPA 53.8%). Combination therapy resulted in strong S-phase arrest where the presence of VPA appears to amplify HU's capacity to slow S-phase to G2 progression. Figure 3B illustrates that the percentage of cells located in S-phase at 24 hrs combination therapy is consistent with the fraction of cells determined to be necrotic (sub G1) following 72 hrs drug exposure. It is previously described that HU mitigates HDACi induction of the cyclin-dependent kinase inhibitor p21 protein enabling cooperative initiation of S-phase driven apoptosis in cancer cells [23]. This trend was observed in the OCI-AML3 cells (Figure 3C). The capacity of VPA to induce p21 and simultaneously arrest cells in G1 increases significantly over time. To assess whether VPA had the capacity to protect cells from HU driven apoptosis, cells were pretreated with HU (100 μM) or VPA (1 mM) for 24 hrs prior to addition of the second complementary compound for a further 48 hrs. Cell viability was assessed by Annexin-PI flow cytometry. Strikingly, when pretreated

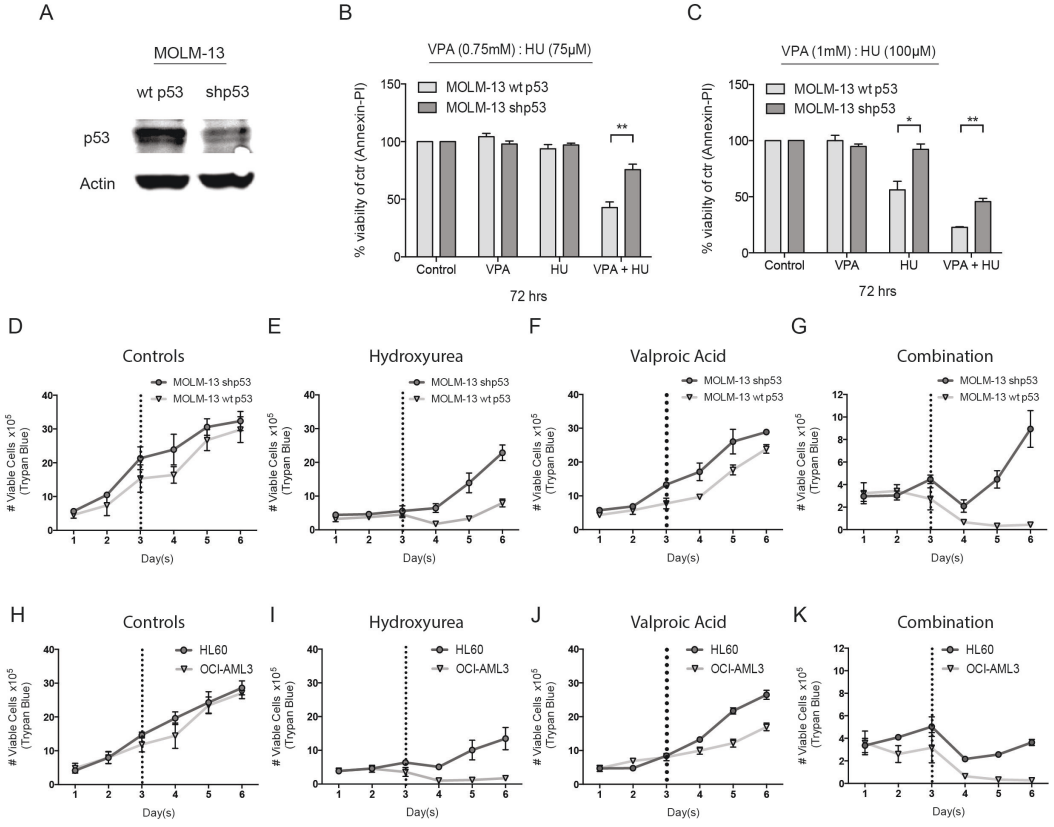

Figure 2: Investigating the role of p53 in HU and VPA combination therapy. (**A**) Lysate was produced from untreated MOLM-13 wt and shp53 cells. Immunoblotting was performed with antibodies targeting p53 and actin. $N = 3$. (**B**) + (**C**) MOLM-13 wt and shp53 cells were treated with HU (75 μM and 100 μM), VPA (0.75 mM and 1 mM) and the combination of both for 72 hrs and apoptosis was determined by Annexin-PI to generate dose response curves. Induction of apoptosis was compared between each cell line at particular treatment conditions ($*P < 0.05$, $**P < 0.01$). $N = 3$. (**D–G**) MOLM-13 wt and shp53 cells and (**H-K**) HL-60 and OCI-AML3 cells were treated with HU (100 μM) and VPA (1 mM), the combination or seeded without treatment. Following 72 hrs cells were washed 2 x in sterile saline and reseeded in wells. The cells were followed for a further 3 days and the number of viable cells was determined at 24 hrs intervals throughout the entire experimental course. Cell counts were performed using the Countess™ Automated Cell Counter (Invitrogen). $N = 3$.

with VPA for 24 hrs cells were significantly more resistant as compared to HU pretreatment (Figure 3D). These results suggest that unmitigated entry to S-phase is necessary for synergistic induction of cell death. The indication that S-phase arrest is important to establish the synergistic effect of combination therapy is emphasized when cell cycle status of OCI-AML3 cells is followed over 72 hrs of combination treatment (Figure 3D).

HU and VPA in combination target DNA damage repair proteins mitigating recovery from DNA double strand breaks

To determine whether S-phase arrest was associated with anticipated HU driven DNA double strand breaks (DSBs), we evaluated the expression of various DNA damage repair proteins following exposure to both compounds and in combination. Protein expression was evaluated at 24 or 48 hrs to ensure > 65% of cells were viable. Immunoblotting revealed a marked increase in the DNA DSB indicator protein γH2AX following combination treatment as compared to monotherapies at both 24 and 48 hrs (Figure 4A). Flow cytometry verified these results and demonstrated additionally that the increased γH2AX was due to the phosphorylated version of the H2AX protein (Figure 4B). Interestingly the tumour suppressor protein p53 was unresponsive to HU treatment alone, but as expected expression was significantly increased upon VPA treatment and this was sustained in combination therapy. Chk1 was decreased in VPA monotherapy [9, 30], however expression appeared unaffected in the combinatory treatment, likely due to the increased presence of DSBs. Crucially, the homologous recombination repair protein, Rad51 was reduced in the presence of VPA, both alone and in combination with HU. Furthermore, immunofluorescence staining revealed γH2AX foci formation in the nucleus of cells exposed to HU or combination (Figure 4C). Additional localisation studies verified the formation of the DNA damage repair foci by nuclear imaging of the MRN complex member, Nsb-1, in HU and combination treated cells (Supplementary Figure 1A). Immunofluorescence staining for Rad51 indicated the protein is reduced and restricted to the cytoplasm in VPA treated cells (Figure 4D). The cytoplasmic restriction of Rad51 following VPA treatment was marked, while Rad51 expression in combination treated cells was diffuse and unspecific. Together these results suggest that the capacity of HU to induce DNA DSBs is vastly enhanced by the presence of VPA, likely due to aberrant expression and localization of Rad51.

Cell death studies confirm synergistic capacity of HU and VPA in OCI-AML3 and primary AML blasts

Prior to performing preclinical studies *in vivo*, we wished to corroborate the capacity of HU and VPA to synergistically induce cell death in OCI-AML3 cells and primary AML blasts whilst remaining non-toxic in healthy peripheral blood mononuclear cells. Flow cytometry assessment of Annexin-PI expression was performed to determine induction of cell death. Dose response curves and combination index analysis in OCI-AML3 cells supported the Hoechst 33342 data presented in Figure 1C (Figure 5A + 5B). Primary AML cells from 10 randomly selected AML patients exhibited a range of sensitivity to HU (75 μM) and VPA (0.75 mM) alone or in combination when treated for 24 hrs (Figure 5C, Table 1). Combining the results from all ten patients demonstrated a significant reduction in mean viability for the HU and VPA combination treatment against either compound alone (Figure 5C). Furthermore, synergism, as calculated by Bliss Independence, was obtained in 9 of the 10 patients samples assessed (Figure 5D). The viability of PBMCs from 4 healthy donors exposed to the HU or VPA alone or in combination for 72 hrs was determined by Annexin-PI staining to reveal no significant increase in cell death (Figure 5E).

The combination of HU and VPA significantly inhibits disease progression in human xenograft models of AML

To evaluate the capacity of combination therapy *in vivo,* the compounds were evaluated in both an OCI-AML3-derived orthotopic model of AML and a primary patient-derived AML xenograft (PDX) (Figure 6). In both models AML cells were engrafted into NOD/SCID IL2rγ^{null} (NSG) mice. The dosing regime for monotherapies and combination treatment was identical in both experiments, though the treatment initiation date differed to reflect differing disease burdens. Preliminary toxicity studies indicated the dosing regime to be well tolerated with minimal adverse effects (Supplementary Figure 2A). The survival curve of the OCI-AML3 orthotropic model illustrates clearly that single agent treatments provoked limited disease response preclinically. Remarkably, a significant increase in survival was observed in combination treated animals compared with controls (p = 0.0003), VPA (p = 0.0001) and HU (p = 0.0014) treated animals (Figure 6A). In a second *in vivo* experiment using an aggressive primary AML PDX model, optical imaging with fluorescently conjugated, multiplexed monoclonal antibodies targeting human leukaemia cells was employed [31, 32]. The purpose of this study was to visualise and quantify the efficacy of the combination *in vivo*. To facilitate imaging therapy was initiated in the stages of advanced disease [31]. *In vitro* studies performed on the primary AML cells used to generate the PDX model confirmed the superiority of the combination compared with monotherapies (Figure 6B). Disease progression before (Day 21) and after (Day 28) treatment was monitored using optical imaging providing

a powerful insight into the therapeutic impact of the treatments employed. Combination therapy significantly reduced the total fluorescence of pooled animals at day 28 compared to both monotherapies (vs. HU $p = 0.0005$, vs. VPA $p = 0.0002$) and controls ($p = < 0.0001$), (Figure 5D and 5E). Combination of HU and VPA at the doses and schedules employed did not adversely affect the body weights or condition in either study (data not shown). Furthermore, despite being treated during advanced stage disease, mice treated with the combination typically survived longer compared with monotherapies and control animals, though this observation was not statistically significant (Supplementary Figure 3A). These data suggest that the combination of HU and VPA can impact upon aggressive AML models of late stage palliative AML therapy. Finally, Figure 6F provides a schematic overview of the mechanism described in the article illustrating the molecular insults attributed to HU and VPA, and how the wt expression of p53 ultimately determines cell death or survival in AML cells.

DISCUSSION

Enhanced efficacy with combination therapy was observed to varying degrees in four AML cell lines (MV4–11, OCI-AML3, MOLM-13, HL-60) compared with single agent treatment (Figure 1). At clinically relevant doses the mutational status of the p53 protein significantly influenced combination efficacy (Figure 1E–1G). The primary AML cells examined were predominantly p53 wild-type and therefore should reflect the general AML patient population (Table 1). The tendency of enhanced combination effect was recapitulated in these samples (Figure 5) but the relatively limited efficacy observed in some wild-type p53 patient cells (I and H) suggests that other factors may also influence therapy response. When comparing MOLM-13 shp53 and MOLM-13 wt p53 cells combination efficacy proved to be significantly more efficient in those cells containing wt p53. Whilst approximately 90% of AML patients are deemed p53 wild-type, a recent study suggests that some

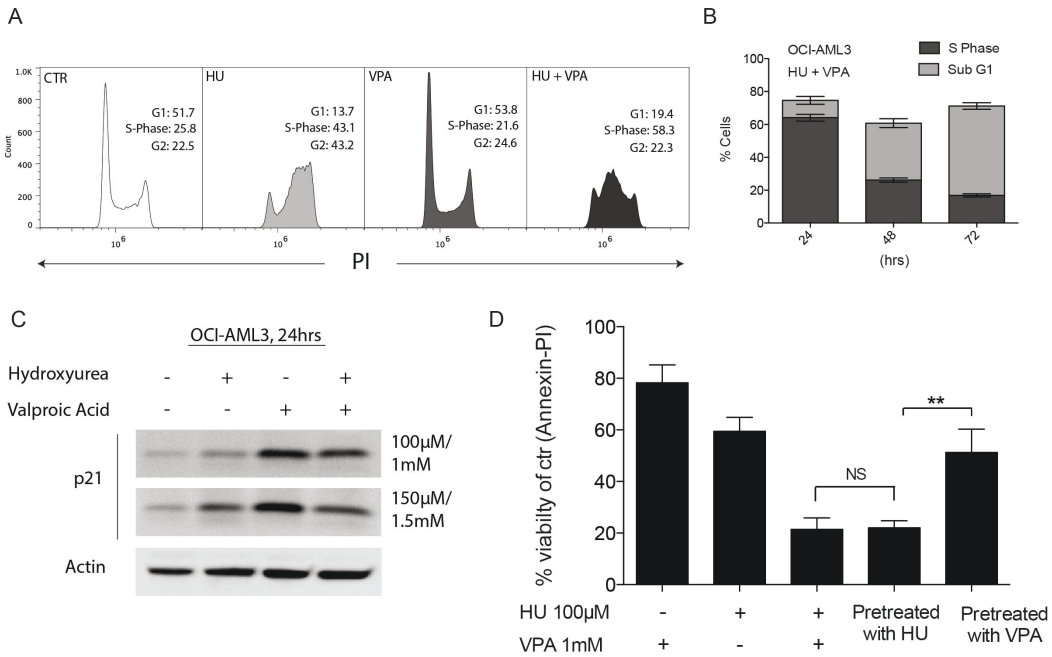

Figure 3: Complimentary regulation of cell cycle status following combination therapy in OCI-AML3 cells. (**A**) OCI-AML3 cells were treated with HU (100 μM), VPA (1 mM) and the combination of both for 24 hrs before cells were stained with PI and cell cycle status analysed by flow cytometry. Analyses was performed in 3 independent experiments with Figure (**A**) providing representative plots. (**B**) Comparison of OCI-AML3 cell populations defined as S-phase or Sub G1 (necrotic) at 24 hrs, 48 hrs and 72 hrs combination treatment with HU (100 μM) and VPA (1 mM). $N = 3$. (**C**) OCI-AML3 cells were incubated for 24 hrs with doses of HU (100 μM and 150 μM) and VPA (1 mM and 1.5 mM). Immunoblotting was with antibodies towards p21 and actin. (**D**) OCI-AML3 cells were treated with HU (100 μM), VPA (1 mM) and the combination of both for 72 hrs. Combination experiments were performed where monotherapies where added for the first 24 hrs with the secondary treatment added for the final 48 hrs of the experiment (Pretreatment with HU/VPA). Apoptosis was determined by Annexin-PI. $N = 3$. The two alternate sequence studies proved to significantly affect the compounds capacity to induce apoptosis (**$P < 0.01$).

of these patients may harbour residual p53 mutant clones that are infrequent and typically undetected [28]. This suggests that in the context of chemotherapeutic regimes, low abundance mutated p53 clones may be selected for expansion. To examine whether the HU and VPA share this therapeutic limitation we performed a treatment recovery assay in MOLM-13 shp53, MOLM-13 wt p53, HL-60 and OCI-AML3 cells (Figure 2). Importantly, wt p53 was revealed to be critical for mitigating cell cycle recovery following combination induced arrest.

It is recognized that HU specifically targets cells during DNA synthesis by depleting the available nucleotide pool [14, 33]. Following 24 hrs of combination treatment cell cycle analysis showed that OCI-AML3 cells were predominantly found in S-phase and therefore vulnerable from potential genotoxicity incited by HU (Figure 3A). In addition we assessed p21 expression during drug exposure. It is reported previously that HU induced down-regulation of p21 may be required for combination driven cell death. Our results confirmed

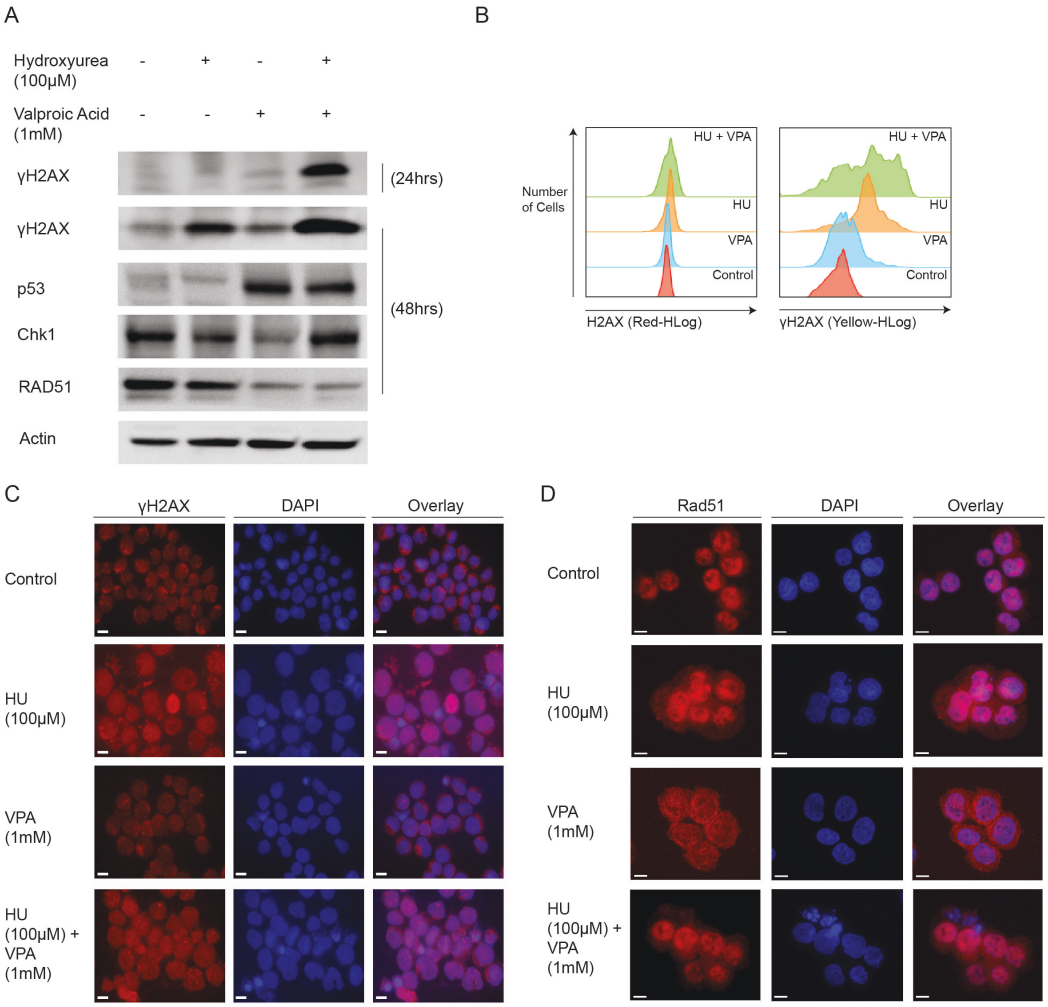

Figure 4: Mechanistic studies demonstrate the combinations capacity to regulate DNA damage repair proteins. (**A**) OCI-AML3 cells were treated with HU (100 μM), VPA (1 mM) and the combination of both for 24 and 48 hrs. Immunoblotting was performed using antibodies towards γH2AX, p53, Chk1, Rad51 and actin. *N* = 3. (**B**) OCI-AML3 cells were treated with HU (100 μM), VPA (1 mM) and the combination of both for 24 hrs. Assesment of H2AX and γH2AX expression was performed using flow cytometry with the Muse™ H2A.X Activation Dual Detection Kit. *N* = 3. (**C** + **D**) OCI-AML3 cells were treated for 48 hrs with HU (100 μM), VPA (1 mM) or in combination, before cytospun, fixed and immunostained for Rad51. Representative image of three independent experiments is shown.

Table 1: Clinical and biological characteristics of the 11 AML patients included in the study

Gender	Age	Previous	FAB	CD34	Cytogenetics	FLT3	NPM-1	P53	Synergy Ranking	Patient ID
M	32	*De novo*	M3	Positive	t(15;17)	ITD	wt	wt	1	G
F	87	*De novo*	M0	Positive	Del 5 (q13, q33)	Normal	nt	wt	2	D
M	78	*De novo*	M4	Negative	+8	Nt	nt	wt	3	C
F	29	*De novo*	M5	Positive	Normal	ITD+Asp835	wt	wt	4	F
M	76	MDS	nt	Positive	Normal	nt	nt	nt	5	B
M	82	*De novo*	nt	Positive	+8	wt	wt	wt	6	A
F	78	*De novo*	M1	Negative	Normal	ITD	Ins	wt	7	E
F	18	*De novo*	M4	Positive	Inv16	wt	wt	wt	8	I
M	64	*De novo*	M5	Negative	Normal	wt	Ins	nt	9	H
M	76	MDS	nt	Positive	Normal	nt	nt	wt	10	J
F	59	*De novo*	M4	Positive	Normal	ITD	Ins	wt	nt	PDX

FAB, French-American-British; F, Female; FLT3, FMS-like tyrosine kinase-3; Ins, Insertion; ITD, Internal tandem duplicate; M, Male; MDS, Myelodysplastic syndrome; NPM1, Nucleophosmin-1; nt, Not tested; PDX, Refers to patient cells used to generate patient derived xenograft model implemented in Figures 6B and 6C; wt, wild type. Synergy ranking determined by the difference in actual vs. expected viability values using bliss independence analysis, 1 = strongest, 10 = weakest (Figure 5D). Patient IDs are chosen arbitrarily for reference to Figure 5C.

the reduced p21 expression in the presence of HU and illustrated that VPA substantially slows passage through S-phase in combination treated cells. By altering the sequence of drug exposure (delaying addition of HU by 24 hours) cells were prevented from entry to S-phase and protected from apoptosis. Together these observations highlighted the importance of the cooperative cell cycle arrest previously attributed to these compounds [23]. They also suggest that when operating synergistically, the compounds depended predominantly on HU driven DNA damage to provoke cell death.

The effects of VPA on leukemic cells are diverse and pleiotropic [34]. The capacity to influence various cellular processes whilst maintaining low toxicity may partly explain its amenity to combination strategies. Previous studies have implicated HDAC inhibitors in the dysregulation of homologous recombination repair (HRR) [35, 36]. Our study confirms this property is operative in VPA treated AML cells. In combination treatment, where cells had incurred substantial DNA DSBs, Rad51 staining appeared reduced, diffuse and absent of the characteristic nuclear foci. Rad51 nuclear foci are associated with the proteins functional role in the repair of DSBs and their presence is considered a valid estimation of HRR capacity [37, 38]. Interestingly, increased expression of Rad51 has been suggested as a mechanism of chemo-resistance in FLT3 mutated AML [37]. HU induced DSBs increase corresponding to dose and drug incubation time [39]. At low concentrations or reduced exposure times, cellular capacity to recover from stalled replication forks is sufficient to avoid incitement of DSBs. This is conveyed by the absence of γH2AX expression flowing 24 hrs treatment with HU, but the subsequent increase at 48 hrs (Figure 4A–4B). However, the dramatic increase of combination-induced γH2AX compared to control and mono-treatments exemplifies the capacity for VPA to enhance the potency of HU.

In vivo efficacy of the drug combination was assessed in two aggressive mouse models of AML (Figure 6) [31, 32]. These studies provided key preclinical indication that repurposing of these compounds as a low-toxic combination therapy may have clinical value in AML. Our second model assessed combination therapy in a patient derived xenograft (PDX) model of AML. Multiplexing of fluorescently conjugated monoclonal antibodies enabled precise imaging of disease progression before and after therapy. We observed no adverse effects in either model upon treatment with the combination of VPA and HU (Supplementary Figure 2A). Treatment of the PDX animals occurred during advanced disease to facilitate imaging thus potentially inhibiting therapeutic efficacy in regards to survival (Supplementary Figure 3A). Nevertheless, *in vivo* imaging provided a clear indication that both the leukemic burden and dissemination of AML blasts was significantly reduced in mice receiving combination therapy when compared to control and mono-therapy groups.

We observed difference in efficiency of HU and VPA in cell line models and a varying effect in the primary AML cells tested *in vitro*, underscoring the future possibility to identify responders or non-responders of HU and VPA based on molecular and biological characteristics of the AML disease. Several case reports [21] together with our animal models have suggested the compounds are unlikely to result in toxicity when administered in combination, indicating the feasibility

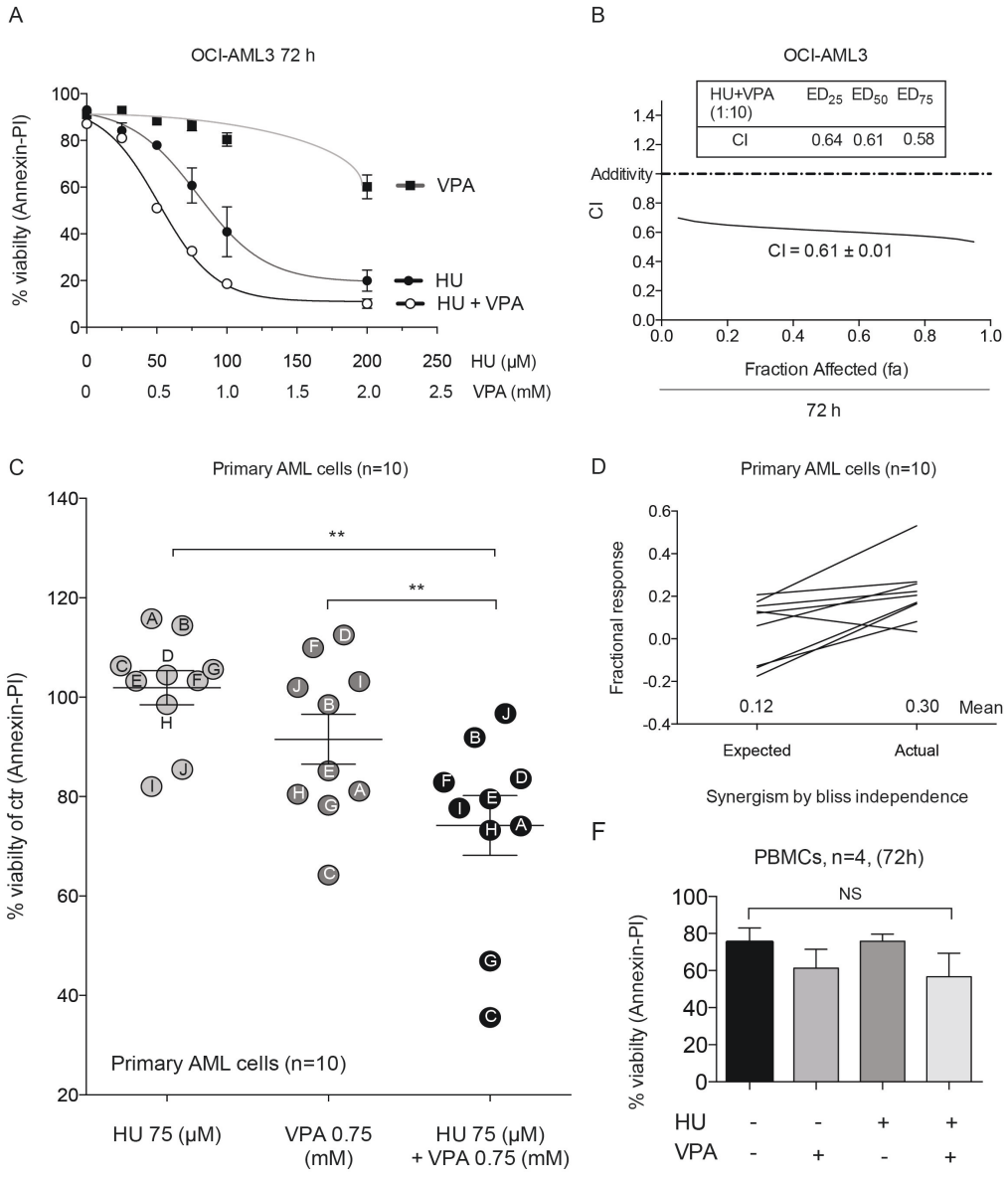

Figure 5: Annexin-PI studies demonstrate synergy in OCI-AML3, primary AML blasts and the non-toxic effect of the combination in PBMCs. (**A**) OCI-AML3 cells were treated with increasing doses of HU (0–200 μM) and VPA (0–2 mM) alone or in combination at a fixed ratio (1:10) for 72 hrs and apoptosis was determined by Annexin-PI to generate dose response curves. $N = 3$. (**B**) The data generated in figure (A) enabled CI vales to be plotted at fa (0–1.0). (**C**) Differences in means of viability between HU (75 μM), VPA (0.75 mM) and combination of both for 24 hrs for the pooled patient data analyzed by Annexin-PI. Results are given as means ± s.e. of mean (**$P < 0.01$, $n = 10$). (**D**) Bliss Independence analysis of expected and actual response for the combinational therapy of HU (75 μM) and VPA (0.75 mM) for each of the individual AML patient samples analyzed by Annexin-PI. (**E**) Peripheral blood mononucleocytes (PBMCs) obtained from four healthy donors were treated with HU (100 μM), VPA (1 mM) and the combination of both for 72 hrs and apoptosis was determined by Annexin-PI. Reduction of viability by the combinational treatment was compared with untreated control cells to reveal a non-significant (NS).

to test HU and VPA in a controlled clinical trial of unfit elderly AML patients. We believe that repositioning of HU and VPA as a combination therapy could significantly enhance the palliative care of patients unsuited to intensive chemotherapy, particularly if non-responders can be predicted prior to initiation of treatment

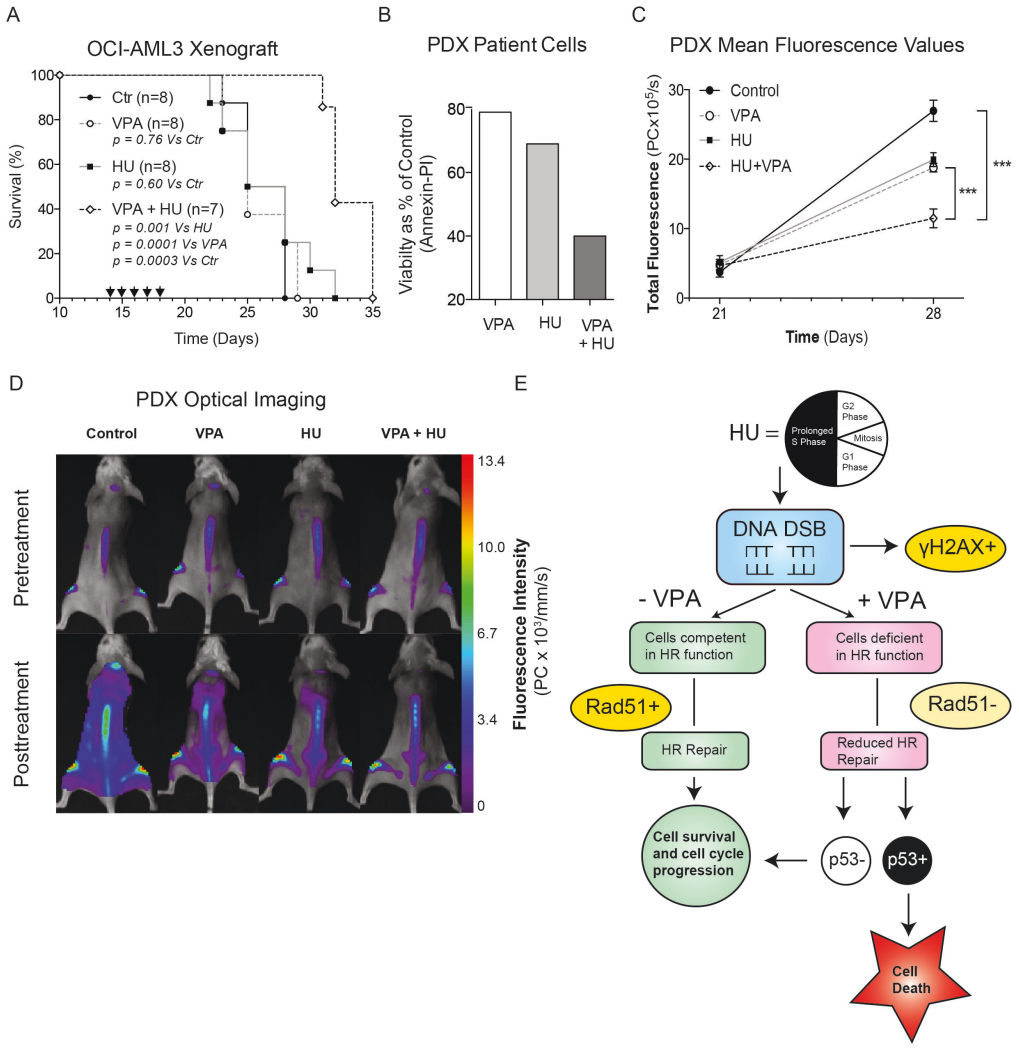

Figure 6: Combinational therapy of HU and VPA represses AML *in vivo*. (**A**) Survival data presented in Kaplan-Meyer curve illustrating the efficacy of HU and VPA and increased survival of combination therapy (log-rank $P = 0.0003$ vs controls, $P = 0.0014$ vs HU, $P = 0.0001$ vs VPA) in the OCI-AML3 orthotopic model of AML. Arrows indicate days on which animals were dosed with both compounds. Control ($n = 8$), HU ($n = 8$), VPA ($n = 8$) and combination ($n = 7$). (**B**) Primary AML cells from the same generation used to generate the PDX model were thawed and treated with HU (75 µM), VPA (0,75 mM) and the combination of both for 24 h and apoptosis determined by Annexin-PI staining. (**C** + **D**) Imaging with multiplexed mAbs was performed before initiation of therapy (day 21) and 1 week later (day 28). Control ($n = 8$), HU ($n = 8$), VPA ($n = 8$) and combination ($n = 6$). Representative images of mice after treatment reveal a significant reduction in total fluorescence (photon count per second) of combination treated mice compared with vehicle controls and monotherapies. Combination compared with control ($p < 0.0001$), VPA ($p = 0.0002$) and HU ($p = 0.0005$). (**E**) Schematic overview of the combination mechanism of action and key molecular events determining cell death or survival in AML cells exposed to HU and VPA. HU, Hydroxyurea; VPA, Valproic Acid; DSB, Double strand breaks; HR, homologous recombination.

MATERIALS AND METHODS

Cell lines, primary AML cells and healthy PBMCs

Seven leukemic cell lines were included in the study, OCI-AML3 (DSMZ), MV4–11, HL-60, KG1-A, THP-1, K562 and MOLM-13 (ATCC). p53 knocked down MOLM-13 cells and empty vector (MOLM-13 shp53) were generated by retroviral transfection for stable expression of shRNA against p53 using the pRETRO SUPER-p53 vector [5]. The concentration of puromycin was steadily increased to 400 µg/ml over a two-week period. Primary AML cells were acquired from patients at Haukeland University Hospital following informed consent. Approval was obtained from the regional Ethics Committee (REK Vest; http://helseforskning.etikkom.no; Norwegian Ministry of Education and Research). Normal peripheral blood lymphocytes were obtained from healthy blood donors (Blood bank, Haukeland University Hospital, Bergen, Norway).

Cell death assays

Evaluation of cell death and apoptosis in cell lines and primary AML cells after drug treatment was performed using Hoechst 33342 staining of nuclear morphology and AnnexinV-propidium idodide (Annexin-PI) detection of apoptosis by flow cytometric analysis. Assays were performed as previously described [5].

Western blotting

Western Blotting was performed as previously described [5]. The following antibodies were used; Actin (sc-4778) Chk1 (DCS-310) p53 (Bp53–12), Rad51 (sc-8349) (all from Santa Cruz Biotech), p21 (EA10, Abcam,), γH2AX (DR1017) and H2AX (DR1016) (both from CalBiochem, Germany). Goat-POD- anti-mouse or anti-rabbit antibody secondary antibodies (Jackson ImmunoResearch) were used as appropriate for primary antibody detection.

Immunofluorescence

Following treatment incubations cells were cytospun onto coverslips followed by fixation and permeabilization with 4% paraformaldehyde for 20 min and ice-cold 99% methanol for at least 20 min at -20 degrees or stored, respectively. Cells were then blocked with 0.5 % Bovine Serum Albumin (BSA) (Roche Diagnostics GmbH) in 1XPBS for 15 min before incubation with primary antibody, rabbit Rad51 (1:200, sc-8349), γH2AX (DR1017, CalBiochem, Germany) or Nsb-1 (D6J5I, Cell Signalling Technology) diluted in 1XPBS with 0.5% BSA at 4°C over night. After three washes in 1XPBS, cells were incubated with secondary antibody (1:5,000 of Alexa 568 goat anti-rabbit (Invitrogen Molecular Probes)) diluted in 1XPBS with 0.5% BSA, was performed in the dark for 1 hour at room temperature. Finally, the coverslip was washed three times with 1XPBS, dipped once in water and mounted in 5 µl Fluoro-gel II with DAPI (Electron Microscopy Sciences, PA, USA). Images of were acquired with a Zeiss Axio Observer Z1 inverted microscope (Carl Zeiss Microimaging GmbH, Germany) and analyzed by the AxioVision 4.8.2 software.

Cell cycle analysis

Cells were fixed in 70% ethanol in PBS overnight. For DNA content analysis cells were pelleted and resuspended in PBS containing 1 mg/ml RNase (Sigma Aldrich) and 10 mg/ml PI, incubated at room temperature for 30 min, then analysed using the Accuri C6 flow cytometer (BD Sciences).

H2AX and γH2AX expression assay

The assessment of H2AX expression in OCI-AML3 cells was performed using the H2AX Muse™ H2A.X Activation Dual Detection Kit (Millipore). 1×10^5 cells were analyzed from each sample condition following 48 hrs drug exposure. Samples were prepared in strict accordance to the manufacturer's procedure and run on the Muse® Cell Analyzer flow cytometer.

Monoclonal antibody conjugation

Monoclonal antibodies (mAbs) CD45 (clone F10–89–4), CD33 (clone WM53), HLA ABC (clone W6/32; all AbD Serotec) were conjugated to Alexa Fluor 680 using the SAIVI Alexa Fluor 680 Labelling Kit (Invitrogen) as described [32]. Protein concentrations of the Alexa Fluor 680–conjugated mAbs degree of labelling were determined using a Nanodrop 1000 spectrophotometer (Thermo Fischer Scientific).

Optical imaging

Prior to imaging, mice were depilated and anesthetized with 1–2% isoflurane (Isoba; Schering-Plough), 0.2 L/min of O_2, and 0.2 L/min of N_2. NIR images were obtained with the eXplore Optix or Optix MX3 Small Animal Molecular Imager system (ART Inc). NIR imaging scans (λ_{ex} = 670 nm, λ_{em} = 700 LP, laser repetition rate 80 MHz, raster scan points 1 mm apart) were obtained 24 hours after administration of Alexa Fluor 680–labelled mAbs (total mAb concentration of 1 µg/g). Optiview software (Versions 1.04 and 2.02; ART Inc) was use to analyse images and perform fluorescence lifetime gating.

Mouse xenograft models

All animal experiments were approved by the Norwegian Animal Research Authority and performed in accordance with The European Convention for the

Protection of Vertebrates Used for Scientific Purposes. Intravenous injection of OCI-AML3 cells (1.5×10^6 cells/100 µL/mouse) and primary AML cells (5×10^6 cells/100 µL/mouse) was performed on female NOD/SCID IL2rγ^{null} (NSG) mice (Vivarium, University of Bergen; originally a generous gift of Dr Leonard D. Shultz, The Jackson Laboratory).

Dosing regime

VPA (100 mg/mL, Orfiril) was sterile filtered from the vial. HU (76.05 g/mol, Sigma

Aldrich) was daily prepared with saline to a concentration of 200 mg/mL.

In both xenograft experiments mice were treated for five consecutive days. In the primary AML model therapy was initiated day 21, whilst in the OCI-AML3 models treatment began day 14. Animals were divided into four groups, control animals receiving 50 µl sterile saline, VPA 350 mg/kg/day, HU 500 mg/kg/day, and combination (VPA 350 mg/kg/day + HU 500 mg/kg/day). All groups contained a minimum of 5 animals and all dosing was performed by intraperitoneal injection.

Statistical analysis

In cell death assays results are displayed as the mean +/− standard deviation. Synergism was calculated by the Chou-Talalay method [40] or by Bliss Independence analysis [41]. In both *in vitro* and *in vivo* studies statistical significance between the averages of varying treatment groups were determined using a two-tailed Student *t* test. *In vivo* survival data was evaluated using the Kaplan and Meier analysis method. A one-way analysis of variance (ANOVA) was performed to ensure no statistical significant difference in weights between the animals in the treatment groups.

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CL and TO: designed and performed research, analysed data and wrote the paper; VA: designed and performed research and analysed data; MM, SK and XNN: performed research; ØB provided primary AML patient cells; BTG: conceived the study and wrote the paper; EMC: conceived the study, designed research, analysed data and wrote the paper.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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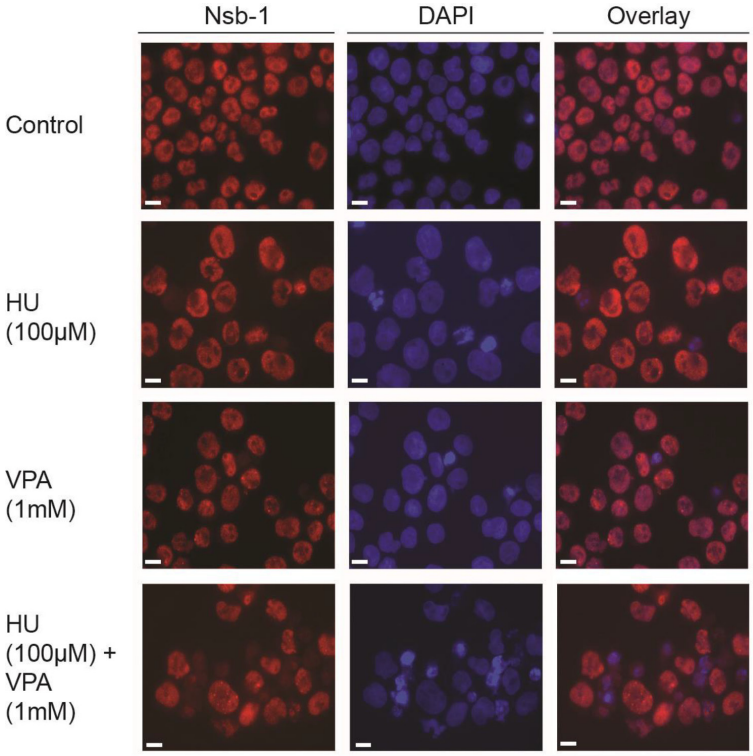
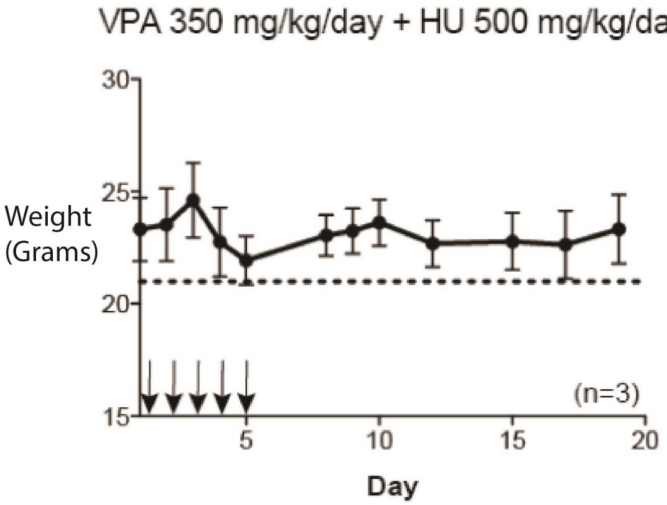
Hydroxyurea synergizes with valproic acid in wild-type p53 acute myeloid leukaemia

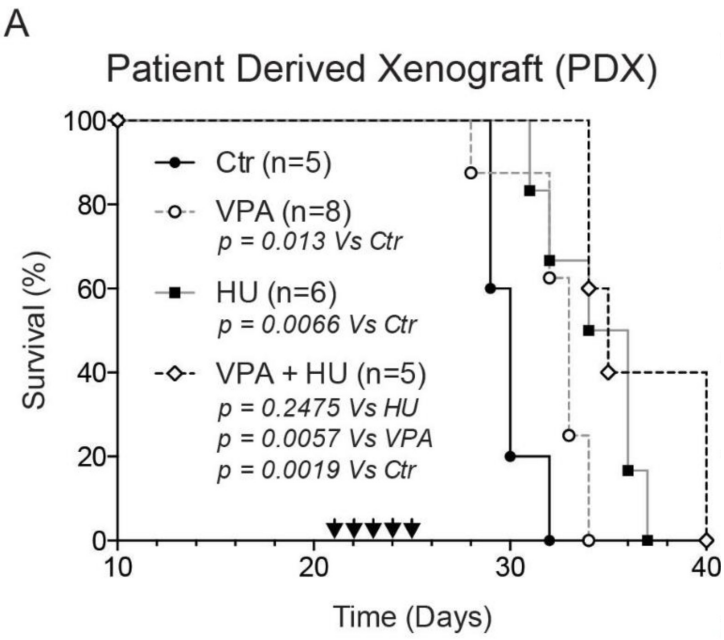
Supplementary Materials



Supplementary Figure S1: (**A**) OCI-AML3 cells were treated for 48 hrs with HU (100 μM), VPA (1 mM) or in combination, before cytospun, fixed and immunostained for Nsb-1. Representative image of three independent experiments is shown ($N = 2$).



Supplementary Figure S2: (**A**) Preliminary animal toxicity data. Weight graphs of female NOD/SCID IL2rγ^{null} (NSG) mice undergoing the 5 day combiation treatment regime administered in leukeamia mouse models. Arrows indiate dosing days. Dotted line represents 10% weight loss and would be considered toxic.



Supplementary Figure S3: (**A**) Survival data presented in Kaplan-Meyer curve illustrating the efficacy of HU and VPA and increased survival of combination therapy for the primary PDX model of AML (log-rank $P = 0.0019$ vs controls, $P = 0.2475$ vs HU, $P = 0.0057$ vs VPA). Arrows indicate days on which animals were dosed with both compounds.

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