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Author(s)	Swords, Ronan
Publication Date	2012-08-15
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**DISRUPTION OF NEDD8 PROTEIN CONJUGATION AS A  
NOVEL THERAPY FOR ACUTE MYELOID LEUKEMIA**

**A thesis submitted to the National University of Ireland in fulfillment of the  
requirement for the degree of**

**Doctor of Philosophy**

**By**

**Ronan Swords MB FRCPI FRCPath**

**Cancer Therapy and Research Center, University of Texas Health Science Center  
San Antonio**

**Thesis supervisors : Professor Francis Giles, Professor Michael O'Dwyer**

**Date of submission : August 2012**

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*Dedicated to the memory of my mother, Margaret Swords, the best person I have ever known.*

## **Acknowledgements**

It is with great pleasure that I write this section of my thesis, not least for being the last bit of writing that will finally conclude it, but more for the opportunity to thank some inspirational people that I have known for a long time, and others that I have just recently come to know since I started this project over four years ago.

My first debt of gratitude must go to my supervisors. I met Mike O' Dwyer early into my training as a hematology specialist registrar and we, both Galway men, our family homes just a stones throw away from one other, fast became good friends. It was through Mike's enthusiasm and inspiration that I became passionate about what I do today. It was also through Mike that I met another Galway man, on a sunny afternoon in Amsterdam, as EHA 2006 was drawing to a close. I knew Frank Giles by reputation and recall anxiously awaiting his arrival in the hotel lobby we had arranged to meet. It wasn't long after this encounter that I would make the move to the US. I owe all of my professional successes in the states, as well as many of my personal ones, to Frank. He was there to provide advice, wisdom, common sense and always a few laughs when the going got tough with this project (which it did on more than one occasion!). Thank you my friend!

I would like to acknowledge Dr. Jennifer Carew for her support and supervision of the pre-clinical experiments in Chapter 2 as well as the help of Ernest Medina with the conduct of the animal experiments. It was my pleasure to have worked with Dr. Meng Wang during his short time at the CTRC and I would like to thank him for his contribution to the first chapter. I remember his patience as I struggled with directions to get to a meeting after work one evening, we never got there in the end but I made up for it by taking Meng to Pappasitos for his first genuine "texmex" experience.

A special thank you to the folks at Millennium pharmaceuticals, who were key in helping with the publication of Chapter 2 and the conduct of the clinical trial in chapter 3. None of this work would have been possible were it not for their support, allowing me access to study this exciting new drug in the laboratory and in the clinic. I would like to acknowledge Steve Blakemore, Michael Milhollen, James Brownell, Allison Berger and James Garnsey for providing assistance with the MLN4924 pharmacokinetic and pharmacodynamic studies. I would particularly like to acknowledge and thank my friend Dr. Bruce Dezube. Bruce has become a close friend and was a great personal and professional sounding board throughout the thesis, always there to provide advice when this project found cause to challenge me. Dr. Peter Smith, who has now left Millennium (good luck to you Pete!) provided help with some of the in vivo data and was always great for troubleshooting when the pre-clinical experiments just wouldn't work right the first time around! Dr. Teresa Soucy was very helpful in sharing some of her thoughts in the preparation of this thesis, Teresa was the first to report the anti-cancer activity of MLN4924 in her Nature paper in 2009. Lastly from Millennium, I want to also acknowledge the help of Dr. Michael Pickard, who was key in providing bio-statistical support for the ASH presentation of our work.

Amongst my clinical colleagues I want to thank and acknowledge Drs. Harry Erba, Dan deAngelo and Bruno Medeiros. I remember learning from the experience and talent of this bunch during advisory boards, meetings and the innumerable conference calls we had together on Tuesday mornings as we began to enroll more and more patients on our trial. I look forward to our paths crossing again in the future and to more successful collaborations. I would also like to acknowledge all the patients that signed consent for this study. Clinical research is impossible without the support and friendship of our patients. I want to pay a special thank you to Daniel Gilbert (who was a real hero on this study and a patient whose life was prolonged and improved thanks to this new agent). Thank you Dan, for making the translational research experience so worthwhile!

To the CRTI group and in particular to my CRTI mentors Drs. Pat Brown, Wendy Stock, Mark Crowther, and Hal Broxmeyer. Thank you for taking the time to listen to my proposals for the future direction of this project, for helping design a great protocol to that end and for reading and critiquing my work. That was a great week in Lajolla, and I hope that many more trainees will benefit from your wisdom and talent!

To my friends that helped support me through this thesis, to Dr. Brian Hennessy, the reason I will always check a slide to make sure it faces the right way up before putting under a microscope, I want to communicate a very personal thank you. We have been through some great times, and some not so great times, since that first morphology session in Letterkenny. Brian's friendship, encouragement, advice and motivation were always there for me to draw on as this project slowly began to take shape. To my good friend Dave, with whom I shared the American adventure, thank you for your common sense and solid advice, delivered at all times, with a sting in the tail! Wouldn't have it any other way though, thanks mate! To Brian, another rock of common sense, thanks for helping get this job done! To Fergal for the laughs and long pondering conversations, to Myles for his thoughts on the design of the thesis, to Alan for his constant encouragement, to Turlough, one of the most thoughtful people I know, to Tony and Cliff (two of my longest friends – we need to arrange dinner at Supermacs soon!) and to anyone I have forgotten to mention, I thank you all!

Lastly, I would like to thank those closest to me. To my girlfriend Megan, for always steering me in the right direction. To my father Brendan, the most noble and honorable man I know. To my mother Margaret, to whom this work is dedicated, I miss you dearly and will always continue to try and make you proud. To my two brothers Mervyn and Connell, thank you both for your patience and understanding when I haven't always been around through the tougher times. I love you all.

Ronan Swords

August 2012

## Abbreviations

<b>AE</b>	adverse event
<b>Abl</b>	Abelson kinase
<b>ALL</b>	acute lymphoblastic leukemia
<b>ALP</b>	alkaline phosphatase
<b>ALT</b>	alanine aminotransferase
<b>AML</b>	acute myelogenous leukemia
<b>AMP</b>	adenosine monophosphate
<b>ANC</b>	absolute neutrophil count
<b>aPTT</b>	activated partial thromboplastin time
<b>AST</b>	aspartate aminotransferase
<b>ASCT</b>	allogeneic stem cell transplant
<b>ATCC</b>	American type culture collection
<b>Bcl-2</b>	B-cell lymphoma -2
<b>Bcl-X<sub>L</sub></b>	Bcl-2 extra long
<b>BCRP</b>	breast cancer-resistance protein
<b>CRL</b>	cullin RING ligase
<b>CR</b>	complete remission
<b>CRi</b>	morphologic complete remission with incomplete blood count recovery
<b>Cul 1</b>	cullin 1
<b>CYP</b>	cytochrome P450
<b>DCF</b>	dichloroflourescein
<b>DLT</b>	dose-limiting toxicity
<b>DNA</b>	deoxyribonucleic acid
<b>ECOG</b>	Eastern Cooperative Oncology Group
<b>FLIP</b>	FLICE inhibitory protein
<b>GCP</b>	Good Clinical Practice
<b>GGT</b>	gamma glutamyl transferase
<b>GI</b>	gastrointestinal
<b>IB</b>	Investigator's Brochure
<b>ICH</b>	International Conference on Harmonisation
<b>I-κB</b>	inhibitory factor-kappa B
<b>IRB</b>	Institutional Review Board
<b>IV</b>	intravenous; intravenously
<b>LDAC</b>	Low dose cytarabine
<b>LIC</b>	leukemia initiating cell
<b>MDS</b>	myelodysplastic syndrome
<b>MLL</b>	mixed lineage leukemia
<b>MRC</b>	medical research council
<b>MTD</b>	maximum tolerated dose
<b>NAE</b>	NEDD8-activating enzyme
<b>NAEβ</b>	NAE subunit NAEβ (also known as UBA3)

<b>NEDD8</b>	neural precursor cell expressed developmentally downregulated 8
<b>NCI CTCAE</b>	National Cancer Institute Common Terminology Criteria for Adverse Events
<b>NF-<math>\kappa</math>B</b>	nuclear factor-kappa B
<b>NPM1</b>	nucleophosmin 1
<b>OS</b>	overall survival
<b>PBMCs</b>	peripheral blood mononuclear cells
<b>PD</b>	progressive disease
<b>P-gp</b>	P-glycoprotein
<b>PK</b>	pharmacokinetic(s)
<b>PS</b>	performance status
<b>SAE</b>	serious adverse event
<b>SC</b>	subcutaneous, subcutaneously
<b>SOD</b>	superoxidase dismutase
<b>TRM</b>	transplant related mortality
<b>Ub</b>	ubiquitin
<b>UBA3</b>	see NAE $\beta$
<b>ULN</b>	upper limit of the normal range

## **Thesis Summary**

Acute Myeloid Leukemia (AML) is a heterogenous disorder with respect to morphology, membrane phenotype, cytogenetics, gene expression profiling and natural history. Clinicians face several challenges in the management of patients with AML, particularly in those who are older where poor prognostic factors are disproportionately over-represented. Adverse cytogenetics, multi-drug resistance and antecedent hematological disorders are all more common. Poorer performance status and medical co-morbidities limit the number of patients fit enough to receive standard therapy and response rates in those that do get treated are suboptimal. Intensive treatment approaches for older patients include anthracyclines with or without the addition of cytarabine, or the use of investigational agents alone or in combination. Since so few patients ultimately receive these treatments the prevailing wisdom is that no standard of care exists for the upfront management of elderly AML. According to Medical Research Council (MRC) data, there have been significant improvements in survival in younger patients with AML during a 30 year period, largely due to the use of more intensive regimens and better supportive care. In older patients treated with intensive chemotherapy over the same period there has been little improvement in survival indicating the need for alternative approaches. Since older patients are living longer and the median age at diagnosis for AML patients in the United States is 67 years, there is an urgent need to find new treatments for these individuals who are traditionally not eligible for most AML trials. Additionally, the abundance of novel agents in oncology drug development mandate the inception of more phase I studies to provide preliminary assessments of these therapies for this disease. The overall aim of this thesis is to explore the activity of a new anti-leukemia agent both in the laboratory and in the clinic, as a means to improve the outcomes for AML patients that are in critical need of more effective therapies. In chapter 1, the controversies in AML management are presented and the role of Nedd8 regulated protein conjugation as a new target for drug development is discussed.

The coordinated balance between the synthesis and degradation of proteins is an important regulator of cancer cell biology. The ubiquitin-proteasome system (UPS) is responsible for the timed destruction of many proteins including key mediators of



fundamental signaling cascades and critical regulators of cell cycle progression and transcription. Within the UPS, the E3 ligases are multi-protein complexes whose specificity is established by their individual components as well as post-translational modifications by various factors including the ubiquitin-like molecule, Nedd8. The Nedd8 activating enzyme (NAE) has been identified as an essential regulator of the Nedd8 conjugation pathway, which controls the activity of the cullin-dependent E3 ubiquitin ligases. The cullins direct the ubiquitination and subsequent degradation of many proteins with important roles in cell cycle progression (p27, cyclin E), DNA damage (Cdt-1), stress response (NRF-2, HIF1) and signal transduction. Considering that Nedd8-mediated control of protein homeostasis is vitally important for the survival of AML cells, we hypothesized that disrupting this process would result in anti-tumor activity. In chapter 2, we test this hypothesis by exploring the pre-clinical profile of MLN4924 (Millenium Pharmaceutical Inc.), a novel first in class small molecule inhibitor of the Nedd8 activating enzyme. We evaluate the anti-tumor effects of this new agent in vitro using AML cell lines and blasts obtained from patients in the clinic. We test the in-vivo effects of MLN4924 in a HL60 xenograft study and propose one of potentially several mechanisms, explaining the sensitivity of AML cells to this compound.

Cytarabine-based therapy has been utilized in AML therapy for more than 30 years. However, the complete response (CR) rates are markedly inferior in older compared to younger patients with AML (45% versus 75%, respectively) due in part, to the reduced ability of elderly patients to tolerate intensive therapy. Improving the outcomes for patients treated with cytarabine-based regimens represents a major clinical challenge in this disease. A randomized study of elderly patients with AML demonstrated that low dose cytarabine (LDAC) is superior to best supportive care. However, this regimen was not associated with any CRs in patients with adverse cytogenetics and/or poor baseline performance scores. Novel approaches are urgently needed to increase the efficacy of LDAC therapy for these patients. Given this, chapter 2 will also describe the results of combination studies of MN4924 and cytarabine, which were conducted as a means to improve the activity of a well established anti-leukemia drug. Finally in chapter 3, we outline the results of our clinical investigation of MLN4924 given to patients with AML and advanced MDS.

# **Chapter 1 : Targeting regulators of protein neddylation in acute myeloid leukemia – a new anti-cancer strategy**

## **1.1 Introduction**

Acute Myeloid Leukemia (AML) is a complex family of disorders and a devastating malignancy that affects all ages. The elegant basic science discoveries in AML that have emerged over the last two decades, now form a springboard for the development of novel new drugs. Fast and affordable ways to sequence the genome or transcriptome of a newly diagnosed patients tumor, offer the prospect of a personalized treatment plan within days of presentation. Despite this and other advances in the field, the challenge in AML therapeutics remains very clear. Conventional treatments for this disease are the same in many of their particulars now as they were three decades ago. Only about 40% of patients considered to have an “intermediate” prognosis will be cured with these drugs [1]. The majority of patients diagnosed with AML will die from their disease therefore new therapies are desperately needed. This chapter will briefly debate the current controversies in the treatment of AML. The need for new therapies will be highlighted with particular focus on protein neddylation as a new target for drug development that has significant promise. This discussion will then serve to provide a framework for the objectives of the thesis, which are listed.

## **1.2 Treatment controversies in AML**

### **1.2.1 Remission Induction**

Standard AML induction protocols have remained essentially the same for the last 30 years. Most newly diagnosed patients will succumb to the disease. The importance of complete remission (CR) has been confirmed again in recent studies. In a large retrospective survey of 879 AML patients who survived beyond 5 years following diagnosis, 801 (91%) achieved CR at some point [2]. Thus, achieving complete remission

has to be the sine qua non for long-term survival. Moreover, patients achieving CR after one cycle of induction, as well as those achieving CR after receiving an identical course of re-induction for residual leukemia on day 14, have identical long-term prognosis, without the need to alter the postremission strategy [3].

### *Young adults*

Using a combination of cytarabine with an anthracycline, 50–75% of patients are expected to enter CR. Intensifying cytarabine doses has failed to improve outcome [3–6]. Higher doses of daunorubicin improved remission and overall survival rates in a recent large prospective Eastern Cooperative Oncology Group (ECOG) study, especially for younger adults [7]. However, the use of this approach as a standard of care is contentious given the response rates observed on the control arm of the study. Additionally, two other randomized studies failed to demonstrate benefit using more intensive anthracycline doses which arguably, could relate to differences in the anthracycline dose and schedule used [8, 9].

### *Older adults*

AML in older patients arises from accumulated abnormalities in myeloid stem cells and as a result, is more likely to overexpress multi-drug resistant proteins, feature an unfavorable karyotype or arise in the context of an antecedent bone marrow failure disorder [10–14]. The results of treatment are inferior to those seen in younger adults and long-term survivors are few. Only 10% to 15% of adults older than 60 years of age enjoy prolonged relapse-free survival after post-remission therapy [15]. Older patients have an increased risk of death during induction, but this should be taken in the context of the early mortality risk from disease complications when given supportive care only. In a landmark study, Harb et al. [16] offered 92 octogenarian and nonagenarian AML patients with no co-morbidities the choice between intensive induction therapy and supportive care. Of these, 59 (64%) patients chose intensive protocols where early death rates on days 7 and 30 were lower than in patients treated supportively (8 vs. 21% and 24 vs. 39%, respectively). In addition, recent data from Swedish and Swiss registries suggest that it is better to treat older patients, even those with poorer performance status, than it is

to give best supportive care [17, 18]. Despite these observations, a sense of nihilism has developed on the part of many clinicians who believe that treating the disease is worse than the disease itself and as a result, a large fraction of older patients with AML are not offered therapy of any type other than supportive or hospice care [19]. Consequently, very few older patients are enrolled on clinical trials which is why optimal induction regimens for fit and unfit patients alike, have yet to be defined.

Selecting which older patients to treat using conventional protocols is challenging. Using clinical scoring systems help to reduce the risk of toxic deaths however [20], even if the right patient is selected for aggressive treatment, the optimal regimen to use is contentious. Complete remission and OS rates with standard induction regimens are poor and a number of intensification protocols have been studied. Remarkably, the Dutch–Belgian Hemato-Oncology Cooperative Study Group (HOVON)/Swiss Group for Clinical Cancer Research (SAKK) reported induction with standard-dose cytarabine and daunorubicin at 90 mg/m<sup>2</sup> for 3 days to be well tolerated in patients older than 60 years of age [21]. Patients of 60–65 years of age who received this intensified induction had higher rates of complete remission (73 vs. 51%), event-free survival (EFS) (29 vs. 14%), and OS (38 vs. 23%) compared with patients in the same age group who received conventional doses. Importantly, the higher dose of daunorubicin was also well tolerated for patients more than 65 years, although a survival benefit could not be demonstrated. The Medical Research Council (MRC) 14 trial [10] also concluded that escalating daunorubicin failed to improve the outcome in older patients; however, lower daunorubicin doses were compared in this trial (daunorubicin 35 vs. 50 mg/m<sup>2</sup> for 3 days) and may not represent the optimal dose ranges for older patients.

If patients are considered unfit for aggressive induction, a variety of “intermediate” intensity therapies are available and work with varying degrees of success. These include monoclonal antibodies (gemtuzumab ozogomicin), alkylators (chlorotazone), and nucleoside analogues (clofarabine, troxacitabine). However, these all produce periods of aplasia lasting 20 or more days and would be preferable to conventional treatment only if the outcome (balance of response and toxicity) were improved in randomized

comparisons [22]. Given these observations, selecting therapy irrespective of performance scores presents a major challenge.

### **1.2.2 Post-remission therapy**

The importance of some form of post-remission therapy has been established [2, 3, 15]. In all AML subtypes, allogeneic stem cell transplantation (ASCT) carries the lowest risk for disease relapse, but carries a transplant-related mortality (TRM) which impacts survival [23]. Historically, physicians were reluctant to offer transplantation, especially if no matched sibling was available. Progress in matched unrelated donor transplantation and the feasibility of reduced intensity conditioning protocols in AML are challenging this conventional wisdom [23].

Despite data from multiple studies of post-remission therapies, significant controversy remains. First, there is no agreement on the number of required cycles of consolidation therapy [23]. Second, the rapid accumulation of newly identified prognostic markers has superseded data from large studies, which did not incorporate these markers. Finally, the availability of modern immunophenotyping and molecular diagnostic techniques may mean that CR patients today, may be ‘better’ patients than complete remission patients a decade ago.

### **1.2.3 Risk stratification**

Cytogenetics are the best established prognostic factor in AML, particularly for some ‘favorable’ [inv16, t (8: 21)] or ‘unfavorable’ (complex karyotype, -7, -5) markers. Recently, data from the MRC and HOVON/SAKK groups shed light on the outcome of patients with trisomy 8, chromosome 3 abnormalities, core binding factor (CBF) leukemias, and monosomal karyotype [24, 25].

In the last few years, a growing number of mutations have been described, but only a few recur in a large number of patients. Frequently, recurring mutations are regarded as ‘drivers’ or significantly associated with a specific outcome and have been reported in oncogenes such as C-KIT, FLT3 [26], NPM1 [27], CEBPA [28], WT1 [29, 30], TET2

[31], MLL [32], IDH1, and IDH2 [33]. Nevertheless, prognostic implications of rare mutations and the relative significance of a single mutation in patients expressing multiple genetic aberrations have still not been determined. For example, a C-KIT mutation was reported to overcome the ‘favorable’ effect of t(8: 21) [34] . Thus, a comprehensive genetic risk stratification algorithm is still pending.

#### **1.2.4 Minimal residual disease**

Contemporary genetic and molecular assays are highly sensitive for the prevalence of minimal residual disease (MRD) at the end of induction. In a large multicenter pediatric study, a reliable MRD marker was identified in 90% of 232 AML patients [35]. MRD higher than 0.1% at the conclusion of induction cycle 2, predicts for a high relapse rate. The 3-year cumulative relapse risk for patients with MRD lower than 0.1% was 20.8%. It should be noted that different MRD markers may have different properties and sensitivity of assays may vary among different laboratories. The emerging concept of a ‘leukemia stem cell/leukemia initiating cell’ (LIC) [36] is fundamental for understanding the association of MRD with relapse risk. LICs are quiescent, thus relatively chemotherapy resistant. LICs consists of 0.1–1% of AML blasts and their low frequencies (1: 100 000 to 1: 10<sup>6</sup> cells) escape the sensitivity of available assays [37]. Thus, inducing a complete remission, and even achieving MRD negativity, does not mean that all LICs are eradicated. Visions for the future include the development of therapies that target pathways which specifically regulate LIC survival and the use of vaccines for patients in first complete remission that enhance endogenous immune surveillance [38].

### **1.3. Protein Homeostasis in Cancer – The NEDD8 Conjugation Pathway**

#### **1.3.1 Introduction**

An improved understanding of how changes in protein homeostasis can drive the pathogenesis of many human diseases has provided the platform for the discovery of several important anti-cancer therapies. The discovery of the ABL kinase as the onco-protein driving proliferation in chronic myeloid leukemia [39, 40] led to the development of imatinib mesylate (Gleevec®, Novartis), the small molecule inhibitor that

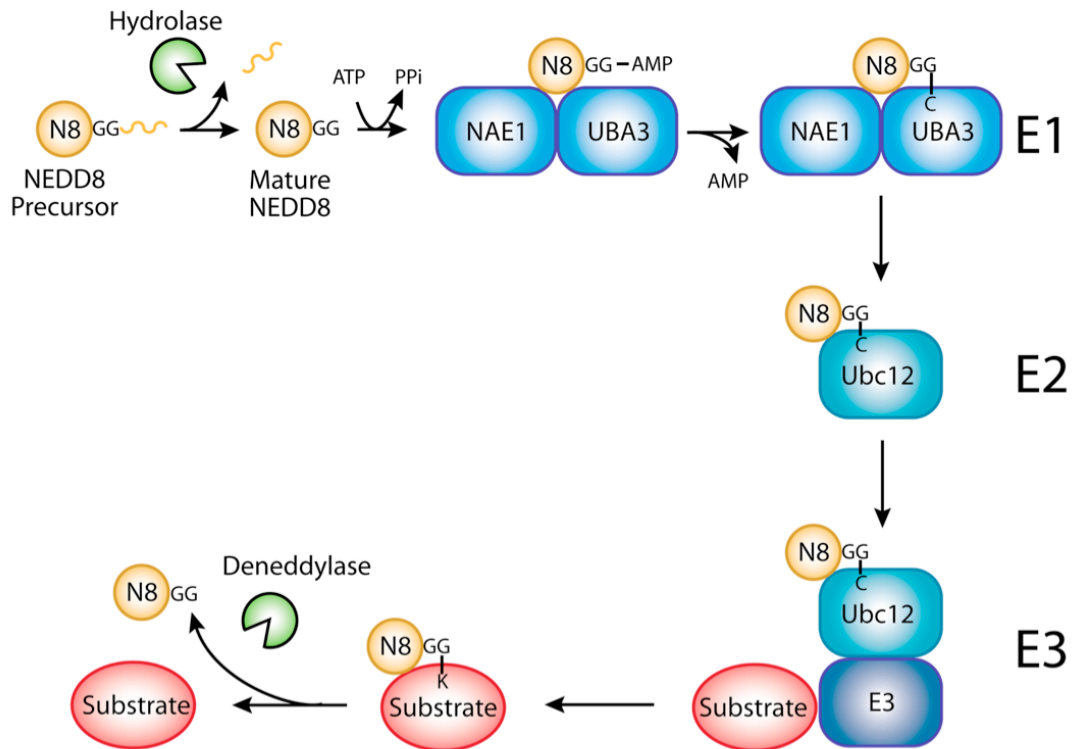
revolutionized experimental therapeutics in cancer [41, 42]. Now, in continuing the era of targeted therapy, a clearer understanding of the NEDD8 conjugation pathway has presented novel targets for the development of new anti-cancer agents. Protein neddylation is catalyzed by E1, E2 and E3 enzymes which conjugate the ubiquitin-like protein NEDD8 (neural-precursor-cell-expressed developmentally down-regulated 8) to a diverse range of target substrates [43-45]. The NEDD8 gene was first described from work conducted on the developing mouse brain where its activity was shown to be down-regulated [46]. Subsequent identification of intracellular substrates conjugated with NEDD8 has provided significant insight into the cellular pathways regulated by this protein. The best-characterized function of NEDD8 is the activation of the cullin-RING family of E3 ubiquitin-ligases (CRLs) which polyubiquitinate proteins, targeting them for degradation by the proteasome. Since many CRL substrates have tumor suppressor activity with important roles in cell-cycle progression, DNA damage and the cellular stress response, preventing the degradation of these proteins could prove to be an effective anti-cancer strategy [47-51]. Of course, targeting protein degradation has already been validated as an effective anti-cancer therapy [52-54] and led to the approval of the proteasome inhibitor bortezomib (Velcade®, Millenium) for clinical use in myeloma and lymphoma [55, 56]. However, this agent lacks specificity in that it prevents the degradation of all polyubiquitin-conjugated proteins which may contribute to clinical toxicity. More selective inhibition of protein degradation may allow for the design of drugs that have similar anti-cancer activity but with improved tolerability. MLN4924, a first in class small molecule inhibitor developed by Millennium Pharmaceuticals, was recently reported as a specific inhibitor of protein neddylation. [48, 50, 57].

### **1.3.2 Processing and conjugation of NEDD8**

Conjugation of NEDD8 to target proteins involves the coordinated and sequential transfer of NEDD8 between a series of enzymes (E1, E2 and E3 enzymes) before being attached to a target substrate (**Figure1.3.1**). NEDD8 is synthesized as an 81-amino acid precursor, which cannot be used in conjugation reactions until hydrolases (UCH-L3 or DEN1) cleave away a portion of the C-terminus to reveal a glycine residue (Gly 76) to which NEDD8 is covalently attached. The processed NEDD8 is initially activated by the NEDD8 activating enzyme (NAE). This enzyme serves as the E1 activating enzyme and

consists of a heterodimer comprising NAE1 (NEDD8 activating enzyme 1) and UBA3 (Ubiquitin-like modifier activating enzyme 3) subunits. NAE activation of NEDD8 is an ATP-dependent reaction which forms a NEDD8-AMP intermediate before NEDD8 is transferred to a cysteine residue in the NAE active site via a thioester bond. The NAE-NEDD8 complex then transfers NEDD8 to an E2 conjugating enzyme. Two of these enzymes have been identified: Ubc12 (also known as UBE2M) and UBE2F [58]. The final step in the process involves the interaction of an E2 with an E3 ligase to conjugate NEDD8 to lysine residues on the substrate protein. The E1 and E2 enzymes that catalyze the neddylation reaction are specific to NEDD8, thus preventing non-specific conjugation with ubiquitin or other ubiquitin-like proteins [43, 59-65]. Several NEDD8 E3 ligases have been proposed to date (**Table 1.3.1**). Deneddylation by NEDD8 isopeptidases like CSN5 (a component of the COP9 signalosome and responsible for cullin deneddylation) and DEN11 (also known as NEDP1 or SENP8) help regulate the loading of NEDD8 on and off protein targets [43].





**Figure 1.3.1 – The neddylation pathway**

The processing and the conjugation of NEDD8 onto its substrate. NEDD8 is initially synthesized as a precursor with a C-terminal tail that is cleaved away by NEDD8 hydrolase. This produces the mature form of NEDD8 which can be conjugated onto substrates via the glycine residues exposed at the C terminus of the NEDD8 molecule. The E1 enzyme, also known as the NEDD8 activating enzyme, a heterodimer formed by NAE1 and UBA3 subunits, binds the mature NEDD8 and an ATP to catalyze the formation of a NEDD8-AMP intermediate. This NEDD8-AMP intermediate then reacts with the thiol group of a cysteine molecule in the active site to form an E1-NEDD8 thioester. This form of the E1 enzyme interacts and transfers the NEDD8 to an E2 enzyme (Ubc12 is depicted in this figure, Ube2F may also be utilized). A specific E3 enzyme recruits the E2-NEDD8 complex and mediates the conjugation of NEDD8 onto a lysine residue of the substrate. In the case of NEDD8 charged Ubc12, NEDD8 may be directly transferred to cullin substrates. The NEDD8 can be removed from the substrate by specific deneddylase enzymes

### 1.3.3 Cellular functions of neddylation

Deficiency of components in the neddylation pathway resulted in a lethal phenotype in several genetic models, thus revealing little detail regarding the role of neddylation in cellular function other than that NEDD8 is essential for embryogenesis and cell viability

[66-71]. The identification of protein substrates that can be modified with NEDD8 eventually enabled a more detailed study of how neddylation affects cellular pathways [80]. In a seminal review of the Nedd8 pathway, Rabut and Peter provide several criteria to determine whether a protein is a Nedd8 target [43]. Based on these criteria, protein substrates should be: 1) covalently attached to Nedd8, 2) detectable under endogenous conditions, 3) dependant on NAE1 and Ubc12 or UBE2F, 4) have a neddylated lysine residue, 5) not be available for attachment to Nedd8 when mutated, 6) be associated with a defined E3 ligase and 7) be associated with deneddylase activity [43]. The currently known neddylated substrates are summarized below (**Table 1.3.1**), however, it should be noted that based on the listed criteria, the only bona fide substrates for Nedd8 are the E3 cullin RING ligases (Cul1, Cul2, Cul3, Cul4A, Cul4B and Cul5). Below we discuss the cullin E3 ligases (as the best characterized neddylated substrates) and p53 (given the relevance of this protein in cancer biology).

#### **1.3.4 Cullin-RING E3 ubiquitin-ligases**

The cullin-RING E3 ubiquitin ligases (CRLs) are a family of E3 ubiquitin ligases consisting of a cullin protein acting as a central scaffold to recruit other subunits to form a modular E3 core complex. An adaptor subunit binds at the N-terminus of the cullin to mediate contact with the substrate recognition protein. The Rbx1 protein containing a RING domain binds to the C-terminus of the cullin to act as a docking site for ubiquitin-charged E2 ligases. However, efficient ubiquitin ligase activity by CRLs also requires a NEDD8 molecule to be conjugated to the C-terminus of the cullin. The addition of NEDD8 in this way is catalyzed through the combined action of Rbx1 and Dcn1 proteins. Neddylation has been proposed to activate ubiquitin ligase activity through several putative mechanisms. NEDD8 arguably could provide a docking site for ubiquitin-activated E2 enzymes (although the evidence for this based on recent structural biology work is weak), induce conformational changes in cullin proteins to bring an E2 closer to its substrate (similarly, the basis for this idea is not clearly substantiated in the literature), promote cullin dimerization and prevent interaction with the E3 ligase inhibitor CAND1 [72]. Several cullin proteins have been described in humans (CUL1, CUL2, CUL3,

CUL4A, CUL4B, CUL5 and CUL7) in addition to 2 cullin-like proteins (PARC and APC). Most of the cullin proteins appear to be neddylated which influence cullin function and substrate selection for degradation [73]. For example, CUL1 catalyzes the ubiquitination of p27, phosphorylated I $\kappa$ B $\alpha$ ,  $\beta$ -catenin, c-myc, c-jun and several others [51]. CUL2 and 5 target HIF1- $\alpha$ , CUL3 targets NRF2 and CUL4A targets CDT1 and p27 [73]. Many of these cullin substrates play critical roles in oncogenesis .

### **1.3.5 The p53 pathway**

In response to cellular stress, oncogene activation or DNA damage, the p53 tumour suppressor activates the transcription of target genes that result in cell cycle arrest, apoptosis or senescence [74, 75]. These important functions of p53 in maintaining genome fidelity are clearly demonstrated by the finding of mutations in this protein in over 50% of human cancers [76]. In AML, p53 mutations/deletions carry a particularly poor prognosis. The stability and activity of p53 is dependent on its post-translational modification by Mdm2, an E3 ubiquitin ligase capable of NEDD8 and ubiquitin conjugation to its target. Polyubiquitination of p53 results in its degradation by the proteasome, and it appears that neddylation also negatively regulates p53 by inhibiting its transcriptional activity, possibly through an interaction between NUB1 and NEDD8 that causes neddylated p53 to become localized to the cytoplasm [30, 45]. Mdm2 auto-neddylation has been reported and may play a role in the kinetics of p53 degradation [30, 77]. Other members of the p53 family are also reported as substrates for neddylation, such as p73, and are regulated in a similar way [78]. The physiologic significance of p53 neddylation remains controversial however, and further work is needed to fully understand the role of Nedd8 in p53 function.

***Table 1.3.1: Proposed neddylation substrates and their functions***

<b>Neddylation protein</b>	<b>NEDD8 ligase</b>	<b>Effect of neddylation</b>
CUL1, 2, 3, 4A, 4B, 5, 7, Parc [73, 79-83]	Rbx1/Dcn1	Enhances E3 ubiquitin ligase activity.
p53 [45, 72]	Mdm2/FBX011	Inhibition of p53 transcriptional activity by localization into the cytoplasm.
p73 [78]	Mdm2	Inhibition of p73 transcriptional activity by localization into the cytoplasm.
Mdm2 [45]	Mdm2	Reduces the inhibitory activity of Mdm2 on p53, whilst another report suggests Neddylation Mdm2 exhibits enhanced stability.
EGFR [84]	c-Cbl	Enhancement of EGFR ubiquitination and its subsequent lysosomal degradation.
AICD [85]	Unknown	Inhibition of AICD transcriptional activity by preventing its interaction with transcriptional co-activators Fe65 and Tip60.
pVHL[62, 86]	Unknown	Promotes interaction of pVHL with fibronectin.
BCA3 [87]	Unknown	Recruitment of SIRT1 histone deacetylase to inhibit NF- $\kappa$ B transcriptional activity.
L11 and other ribosomal proteins [44]	Mdm2	Enhances stability and promotes localization to nucleolus.

### **1.3.6 Rationale for a NEDD8 inhibitor**

Several factors are important for consideration when selecting molecular pathways as targets for anti-cancer drug development. The pathway must be critical for the survival of the cancer cell, it must be subject to mutation or deregulation, disruption of the pathway should not be toxic to normal cells and the pathway must be amenable to pharmacologic manipulation. Given these requirements for rational drug design, the NEDD8 conjugation pathway becomes an exciting pathway for the development of novel agents. Although protein neddylation appears to be critical for some genetic systems [66-68, 70, 71] (which is of concern when exploiting the pathway for therapeutics in humans), pharmacologic deregulation of the NEDD8 conjugation pathway appears to have a selective anti-cancer effect and is well tolerated in early clinical studies, as will be outlined later.

Numerous reports support the importance of the NEDD8 pathway in cancer. Levels of neddylated protein substrates are higher in cancer cell lines when compared to normal fibroblasts and aberrant NEDD8 conjugation in cancer cells has been shown [88]. In addition, transfection of the oral carcinoma cell line HSC4 with a dominant negative Ubc12 which inhibited endogenous neddylation was shown to have anti-proliferative effects, suggesting that aberrantly increased neddylation contributes to the increased cell proliferation seen in cancer cells. Others have shown the association of increased levels of neddylated cullins with high grade neuroendocrine lung carcinomas [89].

Several NEDD8-dependent pathways have been clearly shown to be critical for the growth and survival of a variety of cancer models, many of these pathways are already the focus of drug development. Among these, targeting the NFkB pathway has had the greatest success in the clinic. Drugs like bortezomib and its analogs indicate that there is considerable therapeutic potential in the development of agents capable of more selective inhibition of the ubiquitin-proteasome system. Disruption of the p53/NEDD8 pathway could potentially enhance p53 function, resulting in anti-cancer activity through augmented cell cycle arrest and apoptosis. It is well recognized in p53 deficient mice, that restoration of activity leads to increased senescence and apoptosis [90-92].

However, as we have outlined previously, the link between Nedd8 and p53 is not fully characterized and targeting the Nedd8/p53 pathway clearly requires the presence of wildtype p53, which of course is mutated or non-functional in many patients with cancer which might limit this approach [75].

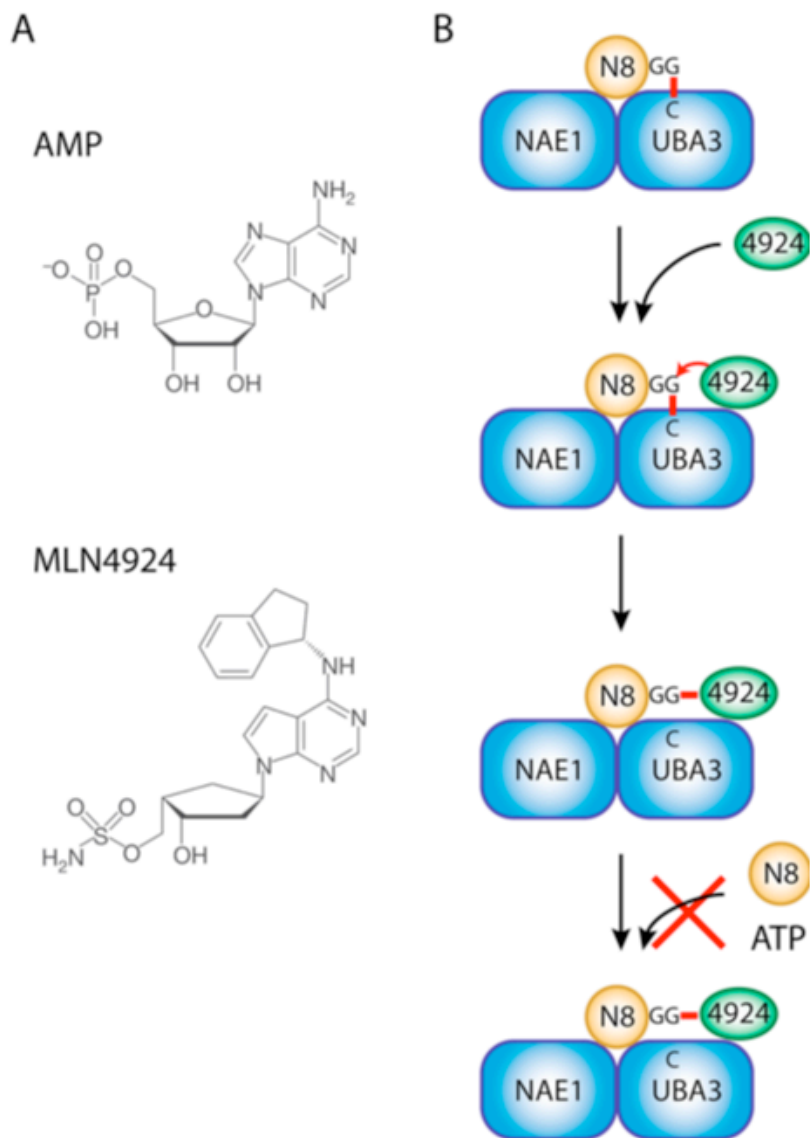
Crystallography studies of the NEDD8 activating enzyme in complex with its substrates, has led to important insights into relevant protein-protein interactions and the catalytic mechanism of the E1 enzyme carrying out the initial neddylation reaction [47, 57]. This has allowed for the design of small molecules that disrupt the interaction of NAE with its binding partners [48, 49]. A neddylation inhibitor could potentially have a better toxicity/efficacy profile as compared to a proteasome inhibitor since proteasome inhibition affects the metabolism of all poly-ubiquitinated proteins as opposed to a neddylation inhibitor that would only affect proteins that are poly-ubiquitinated by CRLs [48]. Since several anti-proliferative proteins (e.g. p27, I $\kappa$ B $\alpha$ ) are tagged with poly-ubiquitin by CRLs which leads to their destruction, a NAE1 inhibitor should increase the levels of p27, I $\kappa$ B $\alpha$  and others thereby blocking cell proliferation [48, 97, 116]. A proteasome inhibitor would not only affect these proteins, but all other regulatory proteins (proteins that are either pro- or anti-proliferative and/or have nothing to do with cell proliferation) as well as house-keeping proteins (protein that need to be turned over due to normal wear and tear) that are destroyed by the proteasome [116]. One could make an argument that a neddylation inhibitor, by virtue of the fact that it affects the metabolism of critical anti-proliferative proteins, would exact a more selective anti-tumor affect than a proteasome inhibitor.

### **1.3.7 NAE as a new target for new drug development**

Ubiquitin and NEDD8 conjugation share similar characteristics. The E1-activating enzymes for both pathways [63] require ATP binding to form an acyl-adenylate intermediate. The resulting Ub-AMP or NEDD8-AMP intermediate then reacts with a cysteine residue in the active site to form an E1-Ub or an E1-NEDD8 complex, before interaction with an E2 takes place. E1 ubiquitin-activating enzymes (UAEs) can be inhibited by hydrolytically stable AMP analogues such as adenosyl-phospho-ubiquitinol,

which bind to active sites and prevent the formation of Ub-AMPs [93]. Due to the size of these analogues, clinical development was not possible since they could not be delivered into cells. Nevertheless, the conservation of the reaction mechanism between ubiquitin and NEDD8 E1 enzymes suggested that a similar strategy could be utilized to design an inhibitor of NAE.

High throughput screening technology discovered N6-benzyl adenosine as an inhibitor of NAE, that with additional iterative medicinal chemistry efforts, resulted in the identification of MLN4924 [48] (**Figure 1.3.2A**). MLN4924 (Millennium Pharmaceuticals) is a potent and highly selective inhibitor of its target in vitro ( $IC_{50}$  4nM) when compared to other E1 activating enzymes [ $IC_{50}$ : UAE (Ubiquitin Activating Enzyme), 1.5  $\mu$ M; SAE (SMO Activating Enzyme), 8.2  $\mu$ M; UBA6 (Ubiquitin Acitvating Modifier Enzyme 6), 1.8  $\mu$ M and ATG7 (Autophagy Related Protein 7), >10 $\mu$ M] or ATP dependant protein kinases [48]. Crystallography data has revealed that MLN4924 inhibits NAE via the formation of a NEDD8-MLN4924 adduct. In the first step of the NAE reaction, ATP and NEDD8 bind with the release of inorganic pyrophosphate. A NEDD8-AMP intermediate is then formed which reacts with cysteine residues in the enzymes active site with the release of AMP. In the last step, a second round of substrates bind and produce a ternary complex where the enzyme has two bound NEDD8 proteins, one as a NEDD8-AMP intermediate and the other as a covalently bound NEDD8 thioester. This species is capable of transferring NEDD8 to an E2 conjugating enzyme via a trans-thiolation reaction. MLN4924 functions by occupying the nucleotide binding pocket of the NAE-NEDD8 thioester and reacts with thioester bound NEDD8 to form an MLN4924-NEDD8 adduct. In this form, no further ATP binding can occur and NAE activation of NEDD8 is disrupted, thereby preventing conjugation with target proteins (**Figure 1.3.2B**) [47]. Unlike the larger AMP analogues necessary for UAE inhibition the MLN4924 AMP analogue is small enough to reach its intracellular target (**Figure 1.3.3**).

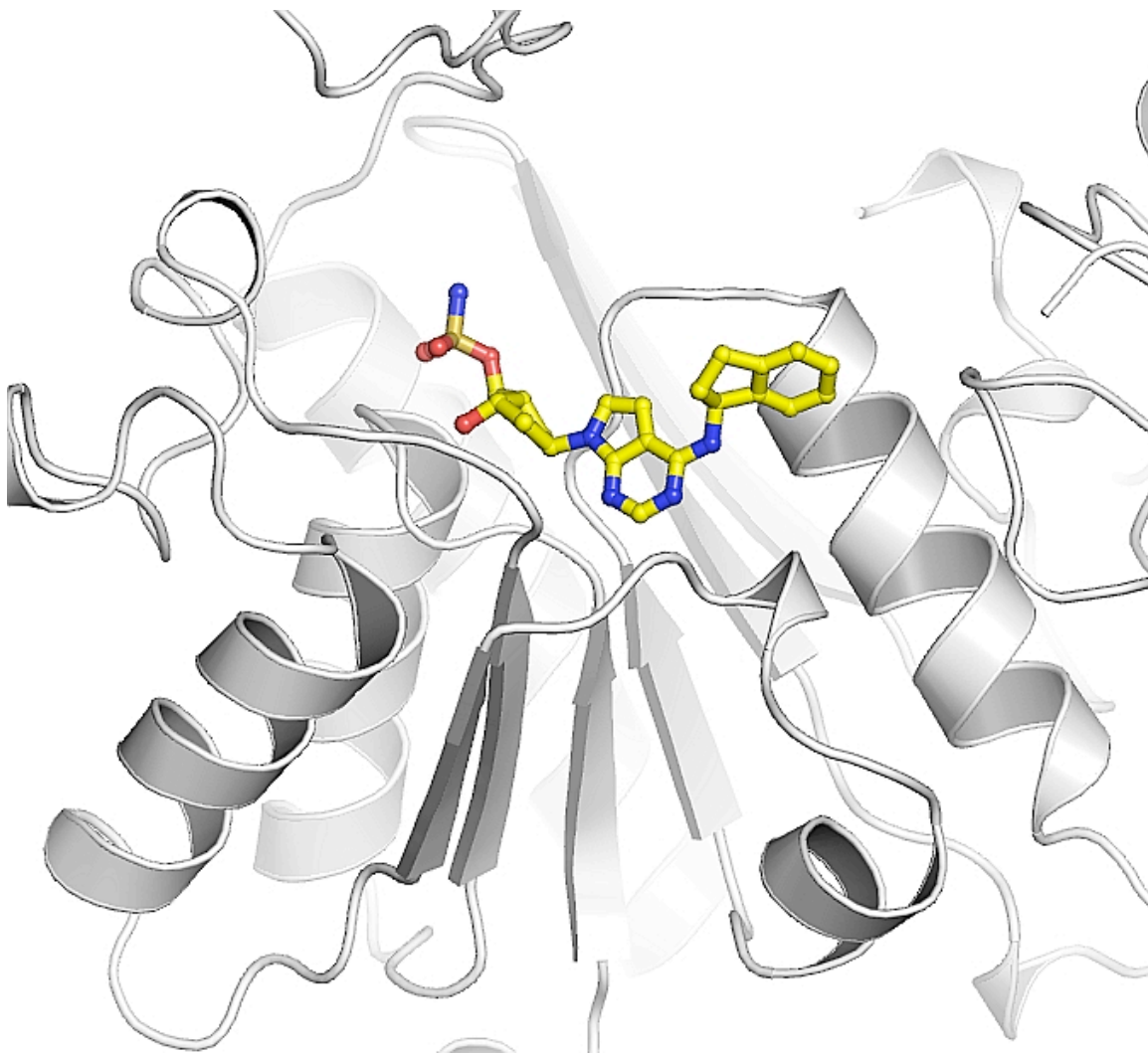


**Figure 1.3.2 – Inhibition of NEDD8 activating enzyme by MLN4924**

**A.** Structure of AMP in comparison to the AMP analogue MLN4924.

**B.** MLN4924 binds to the AMP site in the E1 enzyme and the sulfamate group in MLN4924 attacks the thioester bond between Nedd8 and the cysteine of the E1 making an MLN4924-nedd8 adduct which is stable and inhibits further reactions





***Figure 1.3.3 – MLN4924 target inhibition.***

Structural rendering of MLN4924 occupying nucleotide binding pocket of NAEbeta (Image courtesy of Mike Sintchak, Millennium Pharmaceuticals)

### 1.3.8 Pre-Clinical Studies

We have explored the activity of MLN4924 in pre-clinical AML models, others have documented its activity in other tumor types ( $IC_{50}$ : 0.01  $\mu$ M to 1  $\mu$ M) (**Table 1.3.2**). Administration of MLN4924 to mice bearing xenografts of a variety of solid tumors led to stable disease regression at doses and schedules which were well tolerated [48-50]. Treatment with MLN4924 resulted in a rapid change of neddylation status in vitro and in vivo. Significantly decreased levels of neddylated cullins were observed as early as 30 minutes after exposure to MLN4924 [47, 50, 57] with the subsequent accumulation of cullin-dependent substrates (p27, CDT-1, NRF-2 and phosphorylated I $\kappa$ Ba) but not non-CRL substrates [48-50, 57]. Minimal inhibition of protein turnover in cells treated with MLN4924 was observed when compared to the same cells treated with bortezomib, consistent with the observation that this agent offers more selective disruption of protein degradation [48, 49]. The antitumor activity of MLN4924 has been linked to two mechanisms in pre-clinical models: (1) the induction of DNA re-replication and cell death through dysregulation of Cdt-1 and (2) the inhibition of NF- $\kappa$ B signaling. [50, 57]. The exact mechanisms underlying the cytotoxicity of MLN4924 are likely to be multifactorial and may also depend on the status of other signaling pathways within the cell, which will require further studies to understand more completely.

### 1.3.9 Clinical Studies

As the relevance of the Nedd8 conjugation pathway to cancer biology began to unravel, studies of the clinical grade compound began in hematologic malignancies and solid tumors. Preliminary data has been presented on all these early phase clinical trials.[94-97]. Our pre-clinical evaluation of MLN4924 in models of AML justified the exploration of the clinical activity of this agent in the myeloid neoplasms.

**Table 1.3.2: Summary of effect of MLN4924 on cell lines and murine xenografts**

Origin	Cell line model	50% Growth inhibition by MLN4924 (uM)	Inhibition of tumour xenograft in mice
DLBCL [57]	WSU-DLCL2	0.06, 0.18	Primary human DLBCL
	HBL-1	0.11	
	L1236	0.2	
	U2932	0.03	
	Farage	0.04, 0.05	
	TOLEDO	0.04, 0.07	
	OCI-Ly3	0.15	
	OCI-Ly10	0.01	OCI-Ly10
	OCI-Ly7	0.16	
	OCI-Ly19	0.03	OCI-Ly19
Mediastinal BCL[57]	Karpas 1106P	0.24	
Burkitt's Lymphoma [57]	Daudi	0.03	
	PA682	0.02, 0.06	
	Ramos	0.03, 0.04	
B-cell lymphoma [57]	Pfeiffer	0.01, 0.02	
	RL	0.01, 0.02	
Immunoblastic large cell lymphoma [57]	SR	0.02, 0.04	
Acute myeloid leukaemia [50]	MOLM-13	0.21	HL60
	PL-21	0.21	
	MV4-11	0.21	
	HL-60	0.21	
	Primary AML Cells	0.3-1.0	
Myeloma [98]	NCI-H929	0.27	
	RPMI-8226	0.63	
Colorectal cancer [48, 57]	HCT-116	0.1	HCT-116
Breast carcinoma [99]	MDA-MB-231	0.48	
Lung cancer [48]	Calu-6	0.13	Calu-6
	H460	1.03	H522
Prostate cancer [99]	LNCaP	0.05	
PBMCs [50]		No inhibition at 3.00	

## **1.4. Thesis Objectives**

The beginning of this chapter highlighted the fact that few areas in AML therapy are without debate or controversy. No debate exists over the issue that better treatments for AML are urgently needed. My thesis will address this fact with two objectives:

**Objective 1** – To describe the activity of MLN4924, a novel first in class inhibitor of the Nedd8 activating enzyme (NAE), alone and in combination with cytarabine in pre-clinical models of AML.

**Objective 2** – To translate the laboratory studies of MLN4924 into a phase I clinical trial for patients with AML and related disorders.

## **Chapter 2: The novel NEDD8 Activating Enzyme (NAE) inhibitor MLN4924, has potent activity in pre-clinical models of Acute Myeloid Leukemia**

### **2.1 Introduction**

#### **2.1.1 MLN4924 - A Novel Inhibitor of the NEDD8 Activating Enzyme (NAE).**

MLN4924 (Millenium Pharmaceuticals Inc.) is a first-in-class small molecule mechanism-based inhibitor of NAE, as demonstrated through the formation of a NEDD8-MLN4924 covalent adduct that acts as a tight-binding inhibitor of NAE. It exhibits potent in vitro cytotoxic activity against a variety of human tumor-derived cell lines in which cell death has been shown to correlate with NAE inhibition. In most cancer cell lines studied, including those derived from lung, colon, and lymphoma, the mechanism of cell death appeared to be a consequence of uncontrolled DNA synthesis in S phase followed by a DNA-damage response and induction of cell death [48]. In our AML studies outlined here, we demonstrate that the principal mechanism of apoptotic cell death was through the disruption of cellular redox status [50]. In other studies with the NF- $\kappa$ B dependent lymphoma cell line, OCI-Ly10, apoptosis was observed following a G1 arrest [48], suggesting that there may be multiple consequences of NAE inhibition that lead to cell death depending on the genetic background of the malignant cells. Hematologic and non-hematologic xenograft models support that inhibition of NAE activity may target a broad range of tumors.

#### **2.1.2 MLN4924 – in vitro/in vivo studies**

MLN4924 is a potent and selective inhibitor of NAE activity. It is approximately 300-fold and 1800-fold more selective for NAE over the close family members, ubiquitin-activating enzyme and sumo-activating enzyme, respectively [48]. Moreover, MLN4924 is selective for NAE when compared to kinases and adenosine receptors. In cultured cells, MLN4924 reduced the level of neddylated cullins with a reciprocal increase in known CDL substrates. These effects were consistent with NAE inhibition [48].

Treatment of nude mice bearing subcutaneous HCT-116 human colon tumors with a single subcutaneous (SC) dose of MLN4924 resulted in inhibition of neddylated cullins with stabilization of the CDL substrates, induction of DNA damage, and apoptosis [48]. A single dose of 10, 30, or 60 mg/kg of MLN4924 resulted in sustained levels of stabilized substrate. After repeated dosing of either 2 or 5 consecutive days twice daily (BID), markers of DNA damage and apoptosis were observed in HCT-116 tumors [MLN4924 Investigators Brochure]. MLN4924 was also efficacious when dosed intermittently in a HCT-116 xenograft model. Scheduling studies that examined 30 mg/kg BID treatment regimens (continuous daily dosing, 2 cycles of 5 days of dosing with 5 days of rest, 3 cycles of 2 days of dosing with 5 days of rest, or 3 cycles of 1 day of dosing with 6 days of rest) all showed statistically significant inhibition of tumor growth compared to vehicle ( $p < 0.05$ ). Tumor growth inhibition was similar (ie, not statistically different,  $p > 0.05$ ) for all regimens tested but trended towards greater tumor growth inhibition with more days of dosing. However, MLN4924 antitumor activity was maintained even with intermittent dosing [MLN4924 Investigators Brochure]. In an OCI-Ly10/diffuse large B-cell lymphoma (DLBCL) xenograft model, dosing 2 days weekly for 2 weeks showed similar inhibition of tumor growth to dosing for 5 days out of 21 days. The effects on tumor growth inhibition were also maintained with weekly dosing in this xenograft model [MLN4924 Investigators Brochure].

### **2.1.3 MLN4924 - Pharmacokinetics**

The plasma clearance ( $CL_P$ ) in nonclinical species ranged from relatively low (0.54 L/hr/kg in the chimpanzee), to moderate (1.02 L/hr/kg in the dog and 0.821 L/hr/kg in the monkey), to relatively high (6.05 L/hr/kg in the rat). The plasma half-life ( $t_{1/2}$ ) varied from short (less than 1 hour in the rat) to relatively long (about 15 hours in the monkey). On the basis of the nonclinical findings, MLN4924 was predicted to have a relatively low  $CL_P$  (0.4 L/hr/kg) and a moderate volume of distribution in the terminal phase ( $V_z = 6.5$  L/kg) in humans. The projected human  $t_{1/2}$  (approximately 11 hours) was appropriate for once-daily (QD) intravenous (IV) administration. Preliminary clinical pharmacokinetic (PK) data available from ongoing clinical trials were consistent with the aforementioned predictions. MLN4924 was extensively partitioned into red blood cells (RBCs) in mice, rats, dogs, monkeys, and humans, most likely as a result of the binding to carbonic

anhydrase (CA) in the RBCs. The degree of partitioning was species and concentration-dependent and was saturable.

MLN4924 is predominantly metabolized by the cytochrome P450 (CYP) isozyme CYP3A4, with a small contribution from CYP2D6 (approximately 3%). Therefore, potential for a drug-drug interaction when MLN4924 is co-administered with drugs that are CYP3A4 inhibitors/inducers is expected. Urinary excretion of MLN4924 was negligible in rats, monkeys, and chimpanzees. Studies with efflux pump inhibitors showed that MLN4924 was a substrate for p-glycoprotein (P-gp), breast cancer resistance protein (BCRP), and multidrug resistance associated protein (MRP) 2. MLN4924 was a weak inhibitor of P-gp ( $IC_{50}$  of 41.2 to 56.0  $\mu$ M) and BCRP ( $IC_{50}$  of 6.3  $\mu$ M) but is not likely to be an inhibitor of MRP2 ( $IC_{50} > 200$   $\mu$ M). Based on the projected human plasma maximum concentration ( $C_{max}$ ) value ( $< 2$   $\mu$ M) at the efficacious dose, the potential for drug-drug interaction when MLN4924 is co-administered with a P-gp substrate is predicted to be low.

## **2.2 Project Aims**

**Aim 1** – To investigate the activity of MLN4924 in pre-clinical models of AML

**Aim 2** – To explore the principal mechanisms of action of MLN4924 if in vitro cytotoxicity is shown

**Aim 3** – To evaluate the effects of MLN4924 in a HL60 xenograft study pending the outcomes of aims 1 and 2.

**Aim 4** – To explore the anti-leukemia effects of MLN4924 given in combination with cytarabine

## 2.3 Project Summary

Acute myeloid leukemia (AML) is a disease of the elderly and prognosis is extremely poor with a median overall survival of just 2 months for untreated patients. As such, novel therapeutic strategies are urgently needed to improve clinical outcomes. Considering that Nedd8-mediated control of protein homeostasis is vitally important for the survival of AML cells, we hypothesized that disrupting this process would inhibit proliferation and induce cell death. We tested this hypothesis by investigating the preclinical anti-leukemic activity of MLN4924, a novel first in class small molecule inhibitor of the Nedd8 activating enzyme. MLN4924 induced DNA damage followed by rapid and selective caspase-dependent cell death in AML cell lines and primary AML cells from patients, but not in peripheral blood mononuclear cells from healthy donors. Transient exposure to MLN4924 impaired colony formation in a dose-dependent manner. Kinetic analysis of drug-induced effects on cell cycle distribution revealed that AML cells treated with MLN4924 initially arrested at the G1 transition prior to their subsequent accumulation in the sub-G1 compartment. Assays conducted using MV-411 cells with and without stable shRNA-mediated knockdown of FLT3 expression demonstrated that MLN4924 is highly effective independent of FLT3 status. Further investigation revealed that the activity of MLN4924 was preserved when cells were co-cultured with bone marrow stromal cells indicating that it has the ability to overcome the effects of stromal-mediated survival signaling that has been established to blunt the efficacy of relevant standard of care agents. MLN4924 induced a dose and time dependant increase in the expression of phospho-IkB $\alpha$ , an important target for degradation through the Nedd8 conjugation pathway. The inhibitory effects of MLN4924 on NFkB were confirmed by demonstrating that the transcriptional activity of the NFkB p65 subunit was significantly reduced following drug exposure. Moreover, treatment of immunodeficient mice implanted with HL-60 human leukemia cells with MLN4924 led to an inhibition of neddylated cullins, accumulation of phospho-IkB $\alpha$  and achieved complete and stable disease regression. We showed that disruption of cellular redox status was a critical mediator of MLN4924 induced apoptosis. Our results indicate that MLN4924 is a highly promising novel agent for the treatment of AML and warrants further evaluation in



clinical trials.

Given the multifactorial mechanism of action of MLN4924, and the need to improve the activity of standard therapies, it was hypothesized that MLN4924 would augment the anti-leukemic effects of cytarabine. To test this hypothesis, the combined effects of MLN4924 with cytarabine, on cell viability and apoptosis induction in vitro and in vivo, were evaluated. MLN4924 cooperated with cytarabine to significantly reduce cell viability and induce mitochondrial-dependent apoptosis. The MLN4924/cytarabine combination also promoted increased phosphorylation of the DNA damage response regulator Chk1. In vivo, the combination was well tolerated in a xenograft model. Collectively, these findings suggest that combining the novel NAE inhibitor MLN4924 alone and in combination with cytarabine is a promising strategy in AML and worthy of investigating in the clinic.

## **2.4 Materials and Methods**

### **2.4.1 Cell culture and treatment**

HL-60 and HS-5 cells were obtained from ATCC. MV4-11, MOLM-13, and PL-21 cells were obtained from DSMZ. Primary human AML cells were isolated from the bone marrow of AML patients after obtaining informed consent in accordance with the Declaration of Helsinki. The University of Texas Health Science Center institutional review board approved the collection of peripheral blood and bone marrow specimens. Cells were maintained in RPMI 1640 solution supplemented with 10% fetal bovine serum (FBS), 2nM glutamine, 50IU/ml penicillin and 50 mg/ml streptomycin at 37°C, 5% CO<sub>2</sub> in a humidified incubator. To induce apoptosis, cells with treated with MLN4924 at the concentrations and times specified in the figure legends.

### **2.4.2 Chemicals and Reagents**

Reagents were obtained as follows: MLN4924 (Millennium Pharmaceuticals), antitubulin, antiactive caspase-3, antiphospho- and total I $\kappa$ B $\alpha$ , anti-CDT-1, anti-FLIP, anti-BCL-2, anti-BCL-xL, antiphospho- and total Chk1 antibodies (Cell Signaling), anti-NRF-2 antibody (Santa Cruz Biotechnology), and anti-tubulin (Sigma-Aldrich).

### **2.4.3 Cell viability assays**

Cells were plated in triplicate and treated with defined concentrations of MLN4924 for 72 hours. Viable cells were quantified using the ATPLite assay (PerkinElmer Life and Analytical Sciences). After allowing the kit reagents to equilibrate to room temperature, 50 microL of mammalian cell lysis solution was added to 100 microL of cell suspension per well of a microplate and shaken for five minutes on an orbital shaker at 700 rpm. This step lysed the cells and stabilized ATP. Following this, 50microL of substrate solution was added to the wells and agitated as above. Plates were dark adapted for 10 minutes before measuring color intensity changes at 550nm on a Wallac Victor 1420 Multilabel counter (Perkin Elmer Life Sciences). Cell viability was expressed relative to the absorbance of untreated control cells, which was taken as 100% viable.

### **2.4.4 Analysis of drug induced apoptosis (direct staining – quick method)**

Cells ( $1 \times 10^6$ ) were harvested and spun at 1500 rpm for 5 minutes. After 2 wash steps with PBS, cell pellets were reconstituted with 0.4 ml of fluorochrome solution (propidium iodide 50mg/L in 0.1% sodium citrate plus 0.1% triton X-100). Following a dark adapted incubation period of 1 hour at 4°C, apoptosis was then evaluated by propidium iodide/fluorescence-activated cell sorter (PI/FACS) analysis of sub-diploid DNA content.

### **2.4.5 Colony Assays**

Methocult Methylcellulose medium (80 ml per bottle and containing methylcellulose, FBS, L-glutamine and 2-mercaptoethanol, Stem Cell Technologies Inc.) was adjusted to 100 ml with RPMI 1640 medium. The complete medium was then aliquoted into sterile 15 ml conical tubes and stored at -20°C until use. Cells were then treated with the indicated concentrations of MLN4924 and spun for 5 minutes at 1500 rpm at room temperature before removing the supernatant and re-suspending in fresh RPMI medium. Cells were then plated into wells pre-loaded with methocult medium. Triplicate wells were plated for each condition. Samples were then incubated in a cell culture incubator (at 37°C, 5% CO<sub>2</sub>) for 10-14 days. A stock solution of 0.5% 2,3,5, triphenyl tetrazolium chloride (TTC) in PBS was prepared and stored at 4°C. TTC 0.5% solution was evenly distributed onto each plate (100-150 microL per well) and plates were returned to the

incubator for 2 hours before scoring. Colonies were scored using an Alpha Innotech imaging system. The number of colonies present under each experimental condition was divided by the number of colonies present in the control condition to determine percent survival.

#### **2.4.6 Immunoblot Analysis**

Cells were lysed in buffer and cellular proteins (30 microg) were separated by electrophoresis on 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. After blocking in 5% non-fat milk and 0.05% Tween-20 in PBS, blots were incubated with the antibodies indicated in the figure legends. For detection, appropriate horseradish peroxidase-conjugated secondary antibodies were used. Protein bands were visualized with SuperSignal West Pico Chemiluminescent substrate (Pierce) on X-ray film (Agfa). Beta-tubulin was used to document equal loading.

#### **2.4.7 Stable knockdown of Flt 3**

MV411 cells were pelleted ( $7 \times 10^6$ ) and resuspended in transfection solution alone or transfection solution containing either a small hairpin Flt 3 construct or an empty vector (Dharmacon, Lafayette, CO). Cells were then transfected by nucleofection using the Nucleofector II according to the manufacturers instructions (Amaxa Inc., Gaithersburg, MD). After an incubation of 24 hours, cells were seeded for cell death assays and immunoblotting. Greater than 70% Flt3 knockdown efficiency was confirmed by western blot analysis as indicated in the figures. After confirming efficient knockdown, stable Flt 3 knockdown and control cells were treated with the indicated concentrations of MLN4924 and drug induced apoptosis was confirmed by PI/FACS as described above.

#### **2.4.8 Quantification of NFkB DNA-binding activity**

DNA binding was quantified using the chemiluminescent nuclear factor-kB (NF-kB) transcription factor kit (Pierce Protein Research Products). Kits contained streptavidin-coated 96 well plates with a bound biotinylated-consensus sequence for the p65 transcription factor subunit. These microplates were seeded with cell suspensions treated with the indicated concentrations of MLN4924. The captured active transcription factor was detected with a primary antibody and a secondary conjugated HRP antibody. A

chemiluminescent substrate was then added before measuring color intensity changes at 550nm on a Wallac Victor 1420 Multilabel counter (Perkin Elmer Life Sciences). Relative p65 transcription factor binding was expressed as a percentage of untreated controls.

#### **2.4.9 In vivo evaluation of MLN4924**

HL-60 human leukemia cells were injected into the flanks of nude mice. After tumor growth reached 150 mm<sup>3</sup>, mice were randomly assigned to receive MLN4924 10, 30, 60, or 90 mg/kg twice a day (n = 10 per group), vehicle control (n = 10) for 21 days. Tumor growth and animal toxicities were assessed using calipers and serial weights. The University of Texas Health Science Center institutional animal care and use committee approved the mouse studies.

#### **2.4.10 Reactive oxygen species quantification**

To measure intracellular H<sub>2</sub>O<sub>2</sub>, MV411 cells were treated with the indicated concentrations of MLN4924 and then incubated for 10 min in the dark at 37°C with the H<sub>2</sub>O<sub>2</sub>-sensitive fluorophore 2',7'-dichlorofluorescein diacetate (DCF-DA) (20 μM; Molecular Probes). DCF-DA is hydrolyzed intracellularly to DCF, which is trapped inside the cell. Although DCF-DA itself is not fluorescent, it is oxidized in the presence of H<sub>2</sub>O<sub>2</sub> to the highly fluorescent DCF. Once loaded with the indicator, the cells were harvested in PBS. DCF fluorescence was measured using a FACS analysis with excitation at 488 nm and emission at 530 nm.

#### **2.4.10 Statistical Analysis**

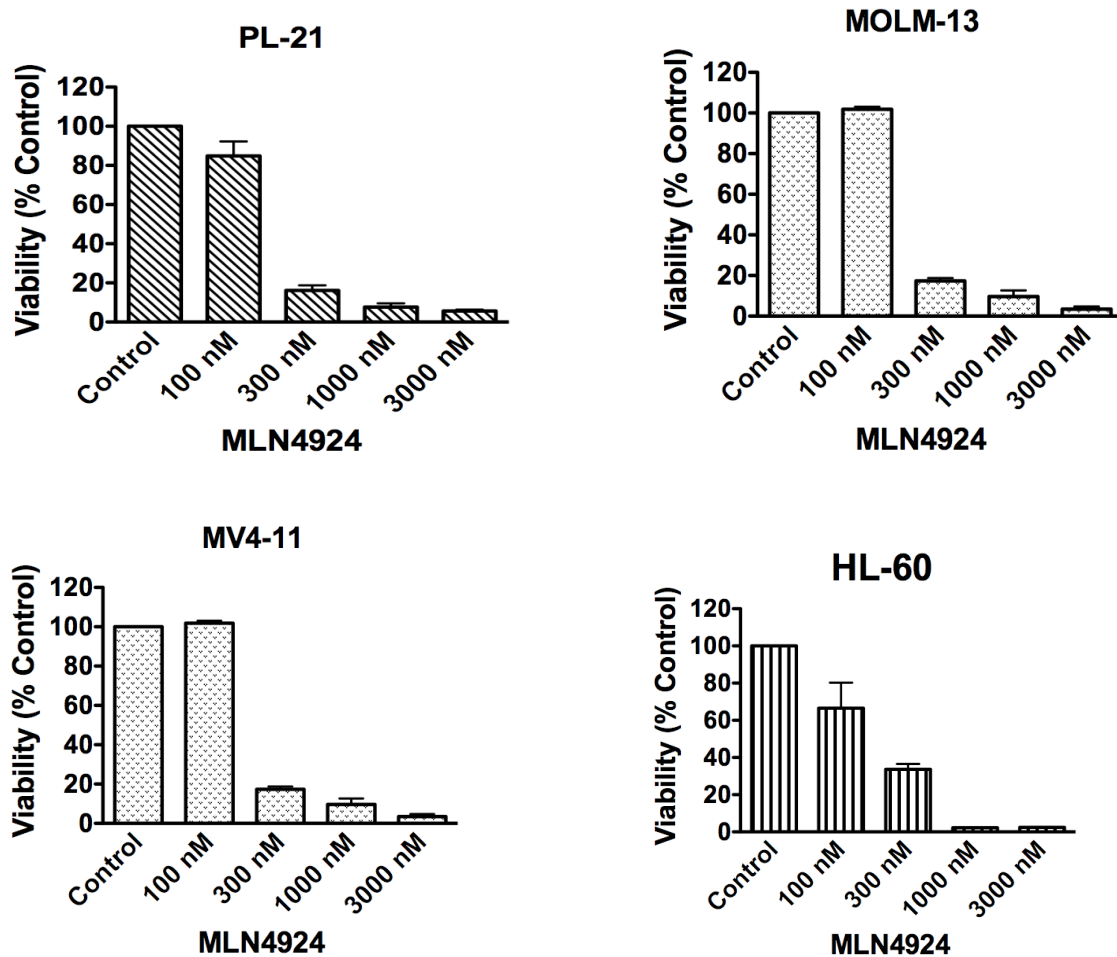
Error bars shown represent the mean values obtained following plating conditions in triplicate +/- 1 standard of deviation. All statistical analysis was carried out using Graphpad Prism 4.

## **2.5 Results**

### **2.5.1 MLN4924 disrupts the viability of AML cells at low nanomolar concentrations**

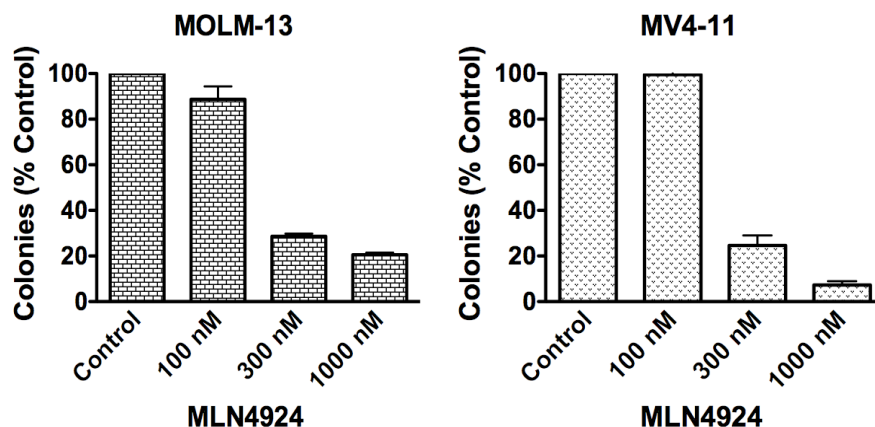
We first assessed the effects of MLN4924 treatment on the viability of AML cells.

Nanomolar doses of MLN4924 (mean 50% inhibitory concentration was 211nM) potently inhibited the viability of MOLM-13, PL-21, MV4-11, and HL-60 cells (**figure 2.5.1**). Low nanomolar concentrations of MLN4924 also impaired the ability of the Flt 3 expressing cells (MOLM-13, MV-411) to form colonies (**figure 2.5.2**).



**Figure 2.5.1 MLN4924 has potent activity in AML cell lines**

MV-411, HL-60, MOLM-13 and PL-21 cells were incubated with the indicated concentrations of MLN4924 and loss of cell viability was confirmed at 72 hours using the ATPlite assay.

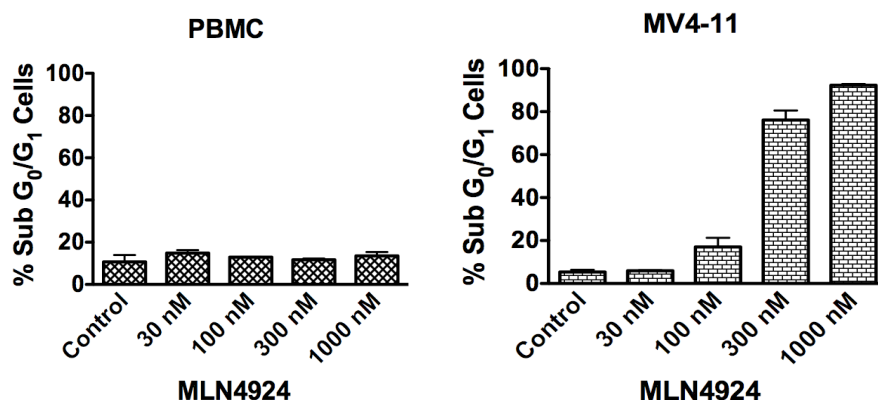


**Figure 2.5.2 MLN4924 inhibits the clonogenic survival of AML cells**

Flt 3 positive AML cells (MOLM-13, MV-411) were treated with the indicated concentrations of MLN4924 for 24 hours. After removal of drug containing media cells were plated with methocult to encourage colony formation. Colonies were scored at day 14 using the Alpha Innotech imaging system

### **2.5.2 MLN4924 has no anti-proliferative effect on normal peripheral blood mononuclear cells derived from healthy donors suggesting a relatively selective anti-leukemia effect.**

After in-vitro activity was confirmed as outlined above, we were interested to see the effects of MLN4924 on normal peripheral blood mononuclear cells (PBMCs). A selective anti-leukemia effect is desirable in new small molecule inhibitors so that rational combinations with approved myelosuppressive drugs can be explored safely in the clinic. Following drug treatment at 1000nM with MLN4924, only 10% of MV-411 cells remained viable at the end of the experiment vs normal PBMCs (100% viable) indicating a selective anti-leukemic affect (**figure 2.5.3.**).

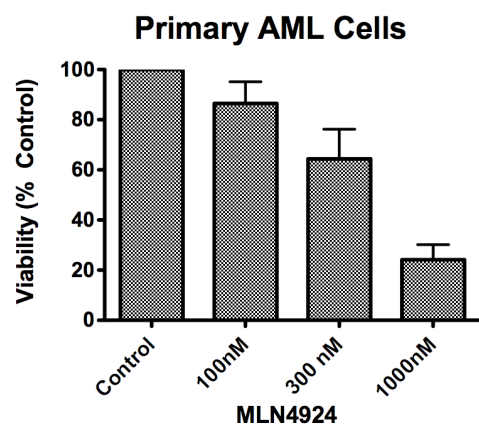


**Figure 2.5.3 MLN4924 has a selective cytotoxic effect on AML cells**

MV-411 cells and normal peripheral blood mononuclear cells from healthy donors were incubated with the indicated concentrations of MLN4924 for 48 hours. Percentages of cells with subdiploid DNA was determined by PI/FACS as described.

### **2.5.3 MLN4924 has equipotent activity in primary patient material across favorable, intermediate and poor prognostic groups**

We next wanted to see if MLN4924 had activity in primary patient material and if so, was this activity preserved across a range of AML risk categories? We showed that in favorable, intermediate risk and adverse risk groups, MLN4924 had documented activity which was preserved across the 3 risk categories however, higher concentrations of drug were required to impair viability in AML blasts from patients compared to cell lines (figures 2.5.4 A & B).



***Figure 2.5.4 (A) MLN4924 causes loss of viability in AML blasts obtained from patients***

Primary cells were obtained from 5 AML patients. Cells were incubated with the indicated concentrations of MLN4924 and cell viability was measured after 72 hours using the ATPlite technique.



Patient	Prognostics	
	Biology	Clinical Outcome
1. Male, 76 years.	Therapy related AML with complex karyotype	Death due to disease progression despite low dose cytarabine, followed by clinical study*
2. Female, 52 years.	Chronic Myeloid Leukemia myeloid blast crisis (CML MyBC) with complex karyotype.	Death due to disease progression despite TKI/3+7** x 1, cytarabine/mitoxantrone x1, clofarabine/cytarabine x 1.
3. Male, 54 years.	AML NOS, normal karyotype.	Death due to disease progression despite 3+7 induction followed by clinical study***
4. Male, 44 years	AML NOS. Normal cytogenetics. NPM1- and FLT3-	CR with 3+7 induction x 1. High dose cytarabine consolidation x 3. Alive in CR.
5. Female, 64 years.	AML with recurrent cytogenetic abnormality, core binding factor leukemia with t(8,21)	Cytarabine/arsenic trioxide x 2, azacytadine/gemtuzumab oozogamicin x 2, clinical study****. Alive with active disease.

**Figure 2.5.4 (B) Primary patient material with clinico-pathologic prognostic indices.**

Patients highlighted in blue had a poor prognosis based on cytogenetics, the patients in orange had an intermediate prognosis and included one patient with a favorable molecular phenotype. The last patient in green had a good prognostic karyotype. AML classification was based on WHO criteria.

\* Phase 1 Study of the Novel MDM2 Inhibitor, AS-1411, in Patients with Refractory Hematologic Malignancies.

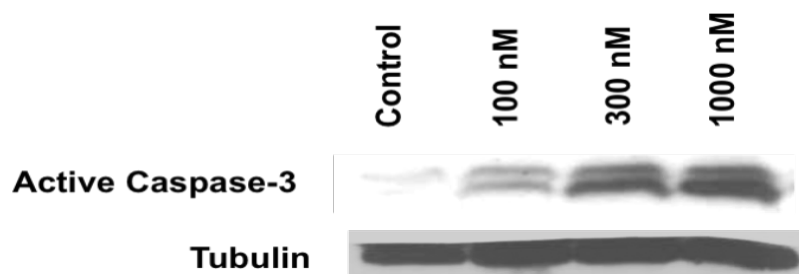
\*\* Tyrosine kinase inhibitor (nilotinib) with conventional idarubicin/cytarabine induction

\*\*\* Phase I/II Study of CP-4055, a Novel Lipid Based Cytarabine, in Patients with Refractory/Relapsed Hematologic Malignancies.

\*\*\*\* Open-Label, Dose Escalation, Phase 1 Study of MLN4924, a Novel Inhibitor of Nedd8-Activating Enzyme, in Adult Patients with Acute Myeloid Leukemia and High-Grade Myelodysplastic Syndrome

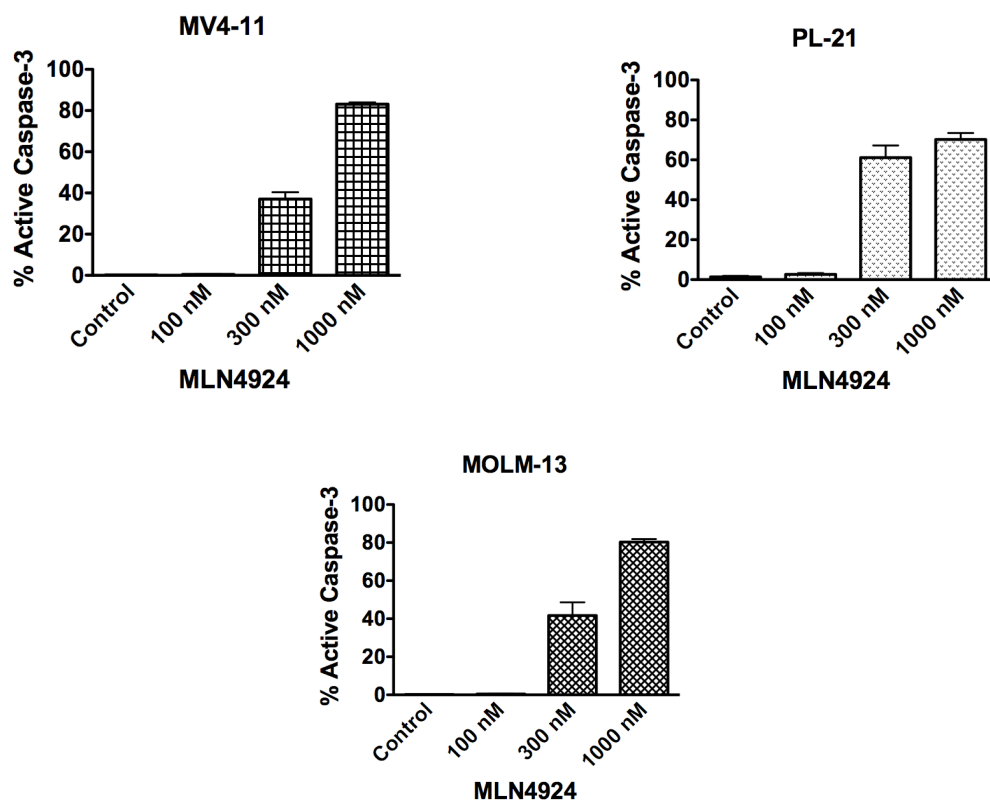
#### 2.5.4 MLN4924 induces cell death by apoptosis in vitro

Given that MLN4924 appeared to have potent and selective activity in both cell lines and primary patient material we wished to explore the mechanisms of cell death following drug treatment. We first evaluated markers of apoptosis (caspase activation and mitochondrial membrane depolarization). Activation of caspase 3 was demonstrated by immunoblotting and flow cytometry. Mitochondrial membrane depolarization was confirmed by flow cytometry (**figures 2.5.5 A, B & C**).



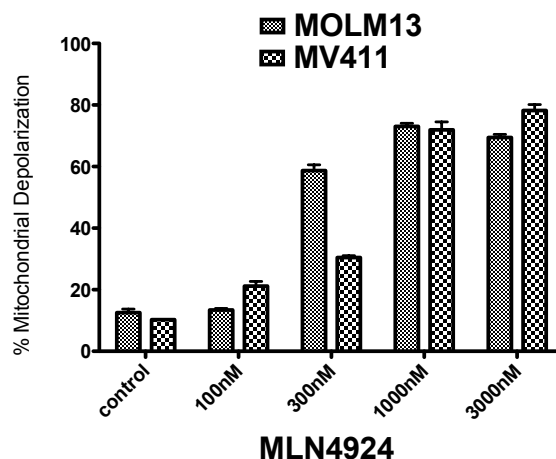
***Figure 2.5.5 (A) Activation of apoptosis following treatment with MLN4924***

MV-411 cells were treated with the indicated concentrations of MLN4924 for 24 hours. Protein lysates were subjected to SDS-PAGE, blotted, and probed with an active caspase-3 specific antibody. Tubulin documented equal loading



**Figure 2.5.5 (B) Activation of apoptosis following treatment with MLN4924.**

MV-411, PL-21 and MOLM-13 cells were treated with the indicated concentrations of MLN4924 and activation of caspase 3 was measured by FACS analysis.

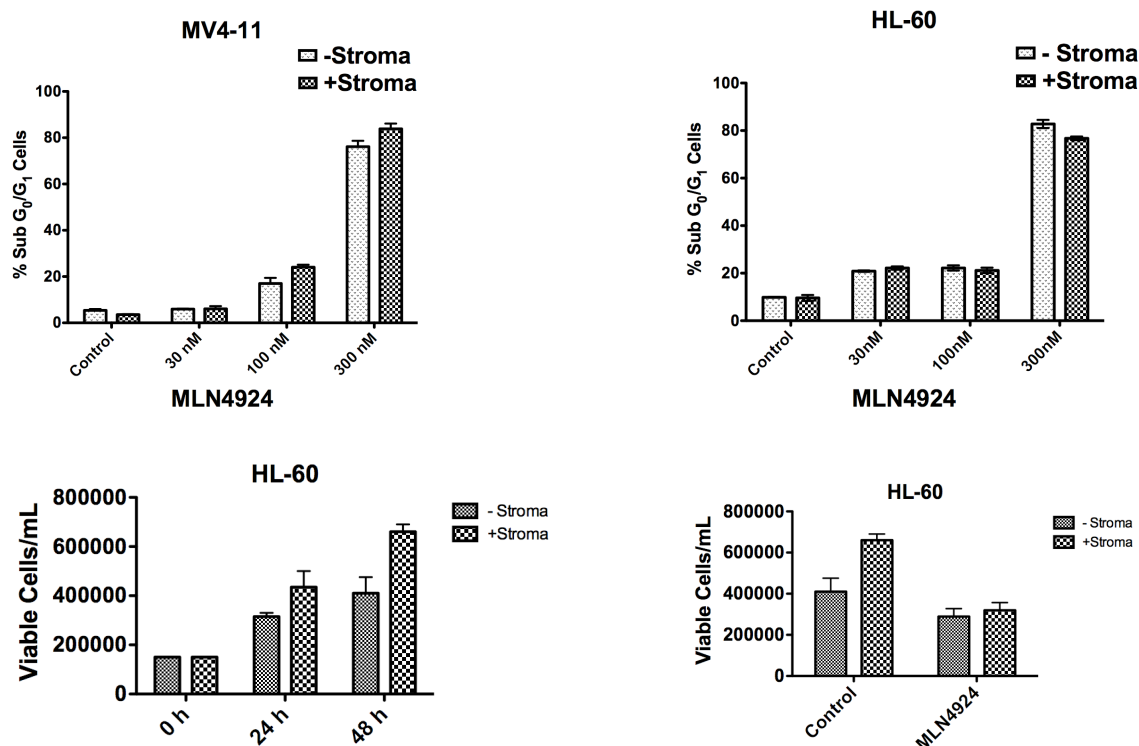


**Figure 2.5.5 (C) Activation of apoptosis following treatment with MLN4924.**

MV-411 and MOLM-13 cells were treated with the indicated concentrations of MLN4924 and depolarization of mitochondrial membrane potential was measured using FACS analysis.

### **2.5.5 MLN4924 overcomes bone marrow stroma mediated survival signals in vitro.**

Following confirmation of drug induced apoptosis in cell lines and primary patient material we next explored the influence of the bone marrow microenvironment on the cytotoxic effects of MLN4924. Drug resistance continues to be a major multifaceted problem that limits successful clinical outcomes for patients with AML. Stromal-mediated survival signaling has been proposed as an important mechanism of resistance to many classes of anticancer agents. Considering this, we investigated the impact of stromal interactions on the sensitivity of AML cells to MLN4924. Co-culturing MV4-11 and HL-60 AML cells with HS-5 human stromal cells did not significantly impact the pro-apoptotic activity of MLN4924 indicating that MLN4924 might potentially overcome the survival advantage provided by stroma in the clinic (**figure 2.5.6**)



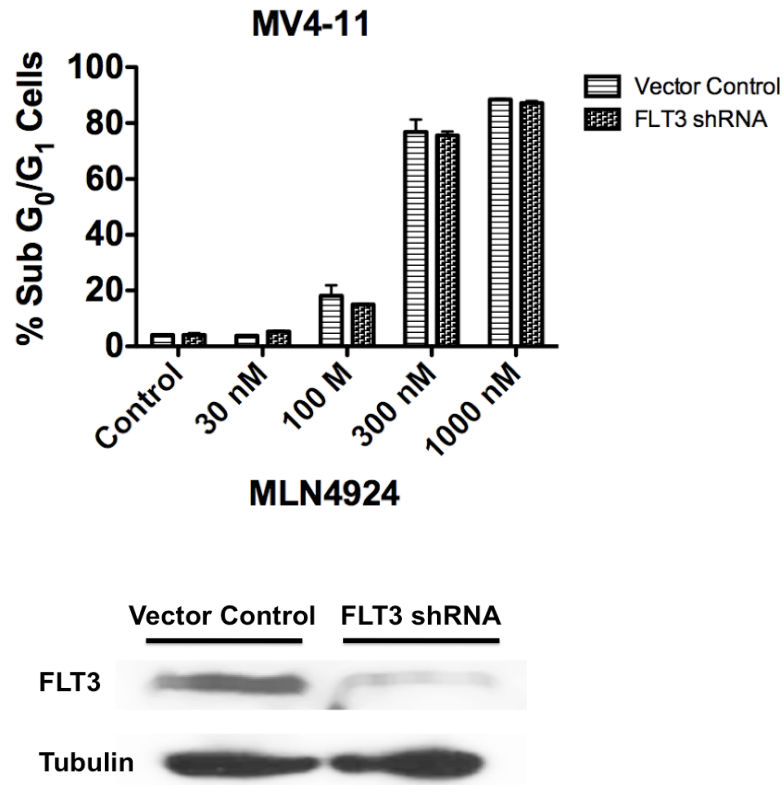
**Figure 2.5.6 Effect of stromal co-culture on the pro-apoptotic activity of MLN4924.**

MV4-11 (upper left) and HL-60 cells (upper right) were treated with the indicated concentrations of MLN4924 for 48h in the presence and absence of HS-5 bone marrow stromal cells. HL-60 cells were incubated with and without bone marrow stromal cells and viable cells were counted after 48 hours by trypan blue exclusion (bottom left). HL-60 cells were treated with MLN4924 with and without the addition of bone marrow stroma and viability was measured by trypan blue exclusion (bottom right). Percentages of apoptotic cells were determined by PI/FACS.

### 2.5.6 MLN4924 activity is preserved independent of Flt 3 expression

In addition to exploring a common extrinsic mediator of drug resistance, we also looked at a common and important intrinsic mediator of drug resistance. The fms-like tyrosine kinase 3 (flt-3) is a receptor tyrosine kinase that is mutated in 30% of patients with AML and confers a bad prognosis in the clinic. We noted that MLN4924 retained its activity in Flt – 3 positive cells (MV-411, Molm-13) and flt-3 negative cells alike (HL-60). We wished to confirm these findings against the same genetic background and to that end, we developed a stable flt-3 knockdown cell line and drug treated knockdown and wild type

MV-411 to evaluate the influence of *flt-3* expression in more detail. We confirmed that MLN4924 retained its effects independent of *flt-3* expression (**figure 2.5.7**).

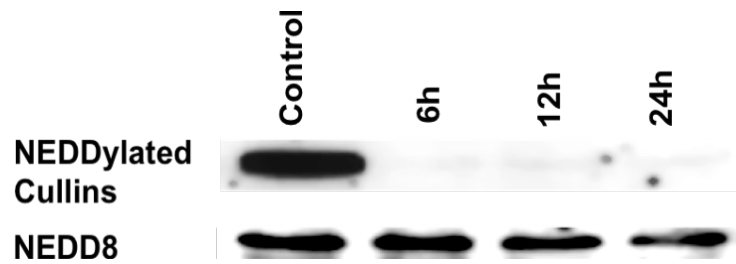


**Figure 2.5.7 Impact of *FLT3* expression on the sensitivity of AML cells to MLN4924.**

MV4-11 cells with and without stable shRNA-mediated knockdown of *FLT3* expression were treated with the indicated concentrations of MLN4924 for 48h. Percentages of apoptotic cells were determined by PI/FACS. *Flt 3* knockdown efficiency was confirmed by immunoblotting as shown.

### **2.5.7 MLN4924 rapidly reduces neddylation of intracellular cullins.**

We next evaluated the effects of MLN4924 on protein homeostasis in AML cells by immunoblotting. Based on medicinal chemistry studies MLN4924 interacts with its target NAE, and isolates the first step of the NEDD8 conjugation pathway. This event reduces the level of neddylated cullins in the cell. We showed that inhibition of NAE activity with MLN4924 produced a time-dependent decrease in the levels of NEDDylated cullins in AML cell lines (**figure 2.5.7**).

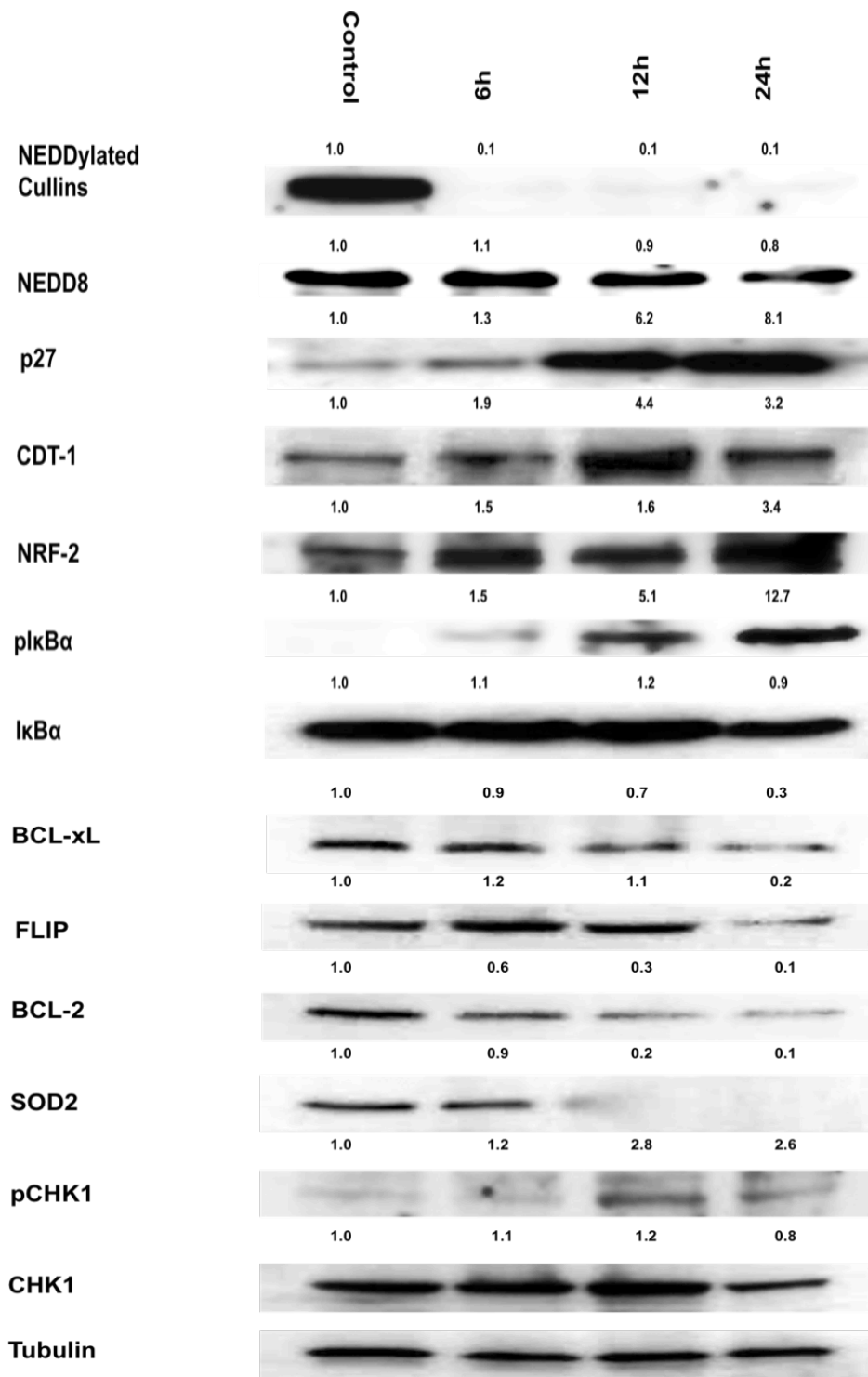


***Figure 2.5.7 Effect of MLN4924 on NEDDylated cullins***

MV-411 Cells were treated with MLN4924 for 24h, lysed, subjected to SDS-PAGE, and probed with NEDD8. Total NEDD8 was used to document equal loading.

### **2.5.8 Stabilization of cullin dependant substrates following MLN4924 treatment**

Following confirmation of pathway inhibition by MLN4924 we evaluated to what extent relevant cullin dependant substrates were stabilized in AML cells. Following rapid reduction of neddylated cullins, several cullin dependant proteins were stabilized including p27, CDT-1, NRF-2, and phospho-I $\kappa$ B $\alpha$ . We also observed the induction of the DNA damage sensor CHK1 (**figure 2.5.8**).



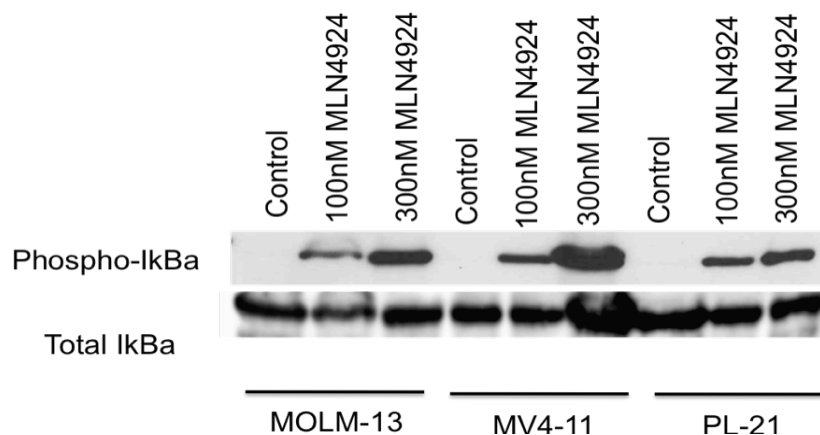
**Figure 2.5.8** *Effects of MLN4924 on NEDDylated substrates and downstream effectors.*

MV-411 cells were treated with MLN4924 for 24h, lysed, subjected to SDS-PAGE, and probed with the indicated antibodies. Tubulin documented equal loading.



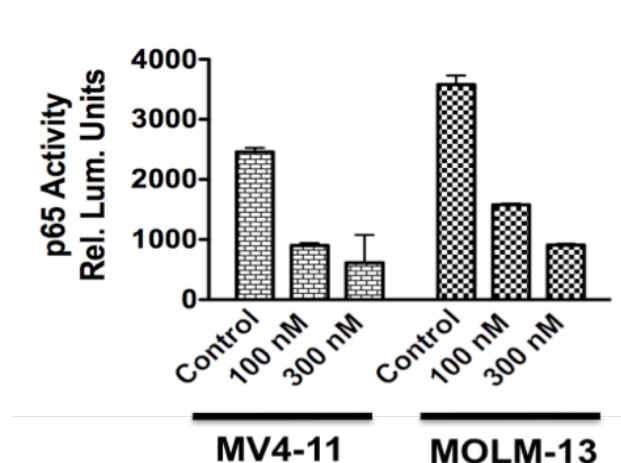
### 2.5.9 MLN4924 reduces the transcriptional activity of the p65 NFκB subunit

Based on the immunoblot studies outlined above, we were interested to note the rapid and pronounced induction of the cullin dependant substrate, pIκBα (**figure 2.5.9 A**). Since this protein is a negative regulator of NFκB signaling, we wished to know the effects of MLN4924 on this pathway and in particular, on the effects of NFκB transcriptional activity. The nuclear factor kappa B (NFκB) transcription factors are constitutively active in many human cancers and regulate the transcription of several genes with fundamental roles in survival signaling and drug resistance. One potential underlying cause of constitutive NFκB activity is the inappropriate degradation of its inhibitor, IκBα. Potentially, through the disruption of NFκB dependant target genes, we could link these events with activation of the caspases and perhaps offer some mechanistic insights into the activity of MLN4924 in AML cells. We hypothesized that MLN4924-mediated IκBα degradation would lead to the inhibition of NFκB transcriptional activity. We show in this experiment, reduced transcriptional activity of the NFκB p65 transcriptional subunit (**figure 2.5.9 B**).



**Figure 2.5.9 (A) Rapid induction of pIκBα following MLN4924**

MV-411, MOLM-13 and PL-21 cells were incubated with MLN4924 for 6 hours and protein lysates were then prepared for western blot analysis.



**Figure 2.5.9 (B) Effects of MLN4924 on NF $\kappa$ B (p65) DNA binding activity.**

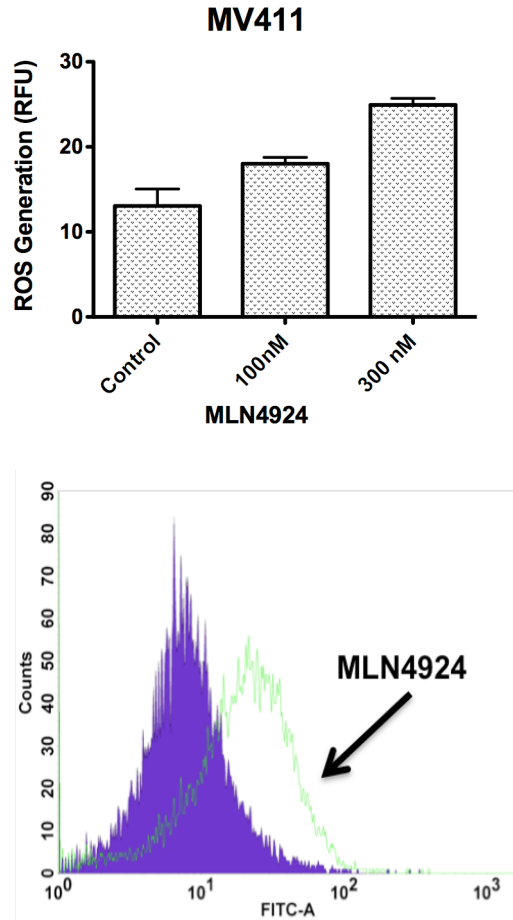
Cells were treated with MLN4924 as indicated for 24h. Relative p65 DNA binding activity was quantified using a chemiluminescent detection method.

#### **2.5.10 MLN4924 reduces the expression of NF $\kappa$ B dependant target genes**

Given the reduced transcriptional activity of NF $\kappa$ B following treatment with MLN4924, we went on to evaluate the activity of NF $\kappa$ B dependant target genes. Treatment with MLN4924 resulted in a time-dependent decrease in the expression of the NF $\kappa$ B targets BCL-2, BCL-xL, FLIP, and superoxide dismutase 2 (SOD2) which we confirmed by western blot analysis (**figure 2.5.8**).

#### **2.5.11 Reduced expression of SOD2 following MLN4924 treatment increases reactive oxygen species in AML cell lines.**

Many types of malignant cells generate significantly higher levels of reactive oxygen species (ROS) than their normal counterparts. This phenomenon can be therapeutically exploited to selectively kill cancer cells by using agents that induce further ROS stress, which culminates in the induction of apoptosis. We hypothesized that the significant decrease in the expression of the antioxidant enzyme SOD2 (**figure 2.5.8**) induced by MLN4924 may result in increased ROS generation. Treatment of MV-411 cells with MLN4924 for 12h led to a significant, dose-dependant increase in ROS generation which was quantified by FACS analysis (**figure 2.5.10**).

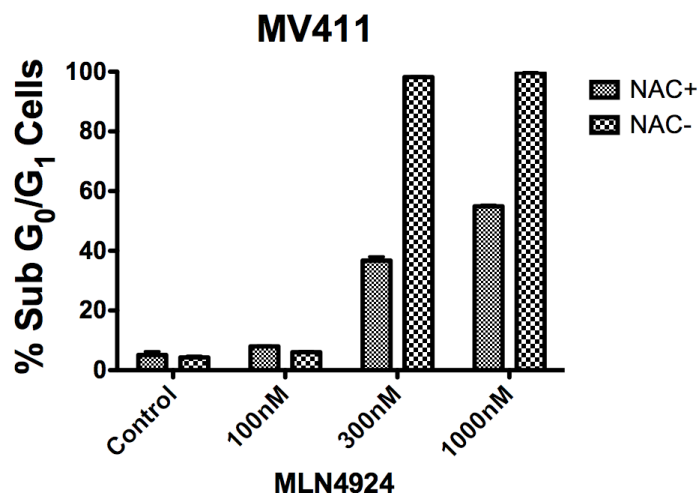


**Figure 2.5.10 Quantification of MLN4924-induced ROS generation.**

MV4-11 cells were treated with MLN4924 for 12h and ROS production was evaluated by staining with dichlorofluorescein (DCF) followed by FACS analysis.

### **2.5.12 Pre-treatment of cells with the anti-oxidant N-acetylcysteine (NAC), significantly abrogates the cytotoxic effects of MLN4924**

To test the contribution of MLN4924-induced ROS production to its anticancer mechanism of action, we evaluated the pro-apoptotic effects of MLN4924 in the presence and absence of the antioxidant N-acetyl cysteine (NAC). NAC treatment significantly blunted the degree of apoptosis stimulated by MLN4924, indicating that ROS production is an important event in MLN4924-induced cell death (**figure 2.5.11**).

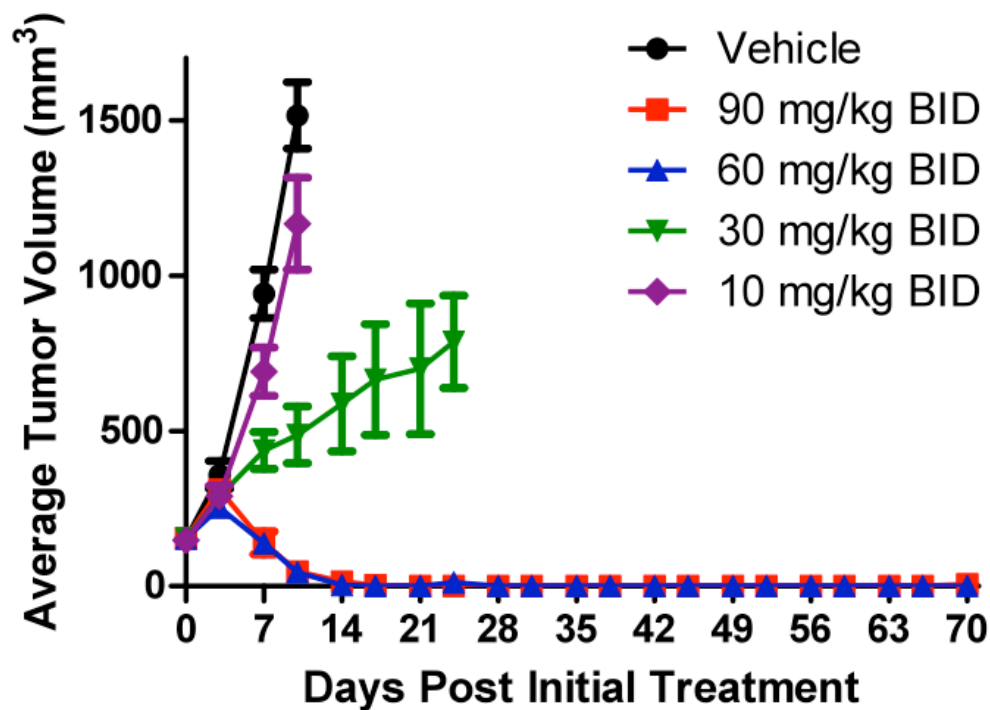


**Figure 2.5.11 Impact of antioxidant treatment on MLN4924-induced apoptosis.**

MV-411 cells were treated with the indicated concentrations of MLN4924 for 48 hours. NAC+ cells were pre-treated with 5uM of the antioxidant N-acetylcysteine for 24 hours prior to drug treatment. Apoptosis was measured by PI/FACS analysis.

### **2.5.13 MLN4924 is well tolerated in a HL60 murine xenograft model and shows significant anti-tumor activity**

Given the in vitro effects of MLN4924 outlined, we were interested in seeing if the anti-tumor effects of MLN4924 could be reproduced in an vivo model. We evaluated the anticancer activity of MLN4924 by administering MLN4924 or vehicle controls to mice implanted with HL-60 xenografts. MLN4924 treatment led to a dose-dependent decrease in disease burden and 10/10 animals in the groups treated with 60 and 90 mg/kg experienced stable regressions (**figure 2.5.12**).

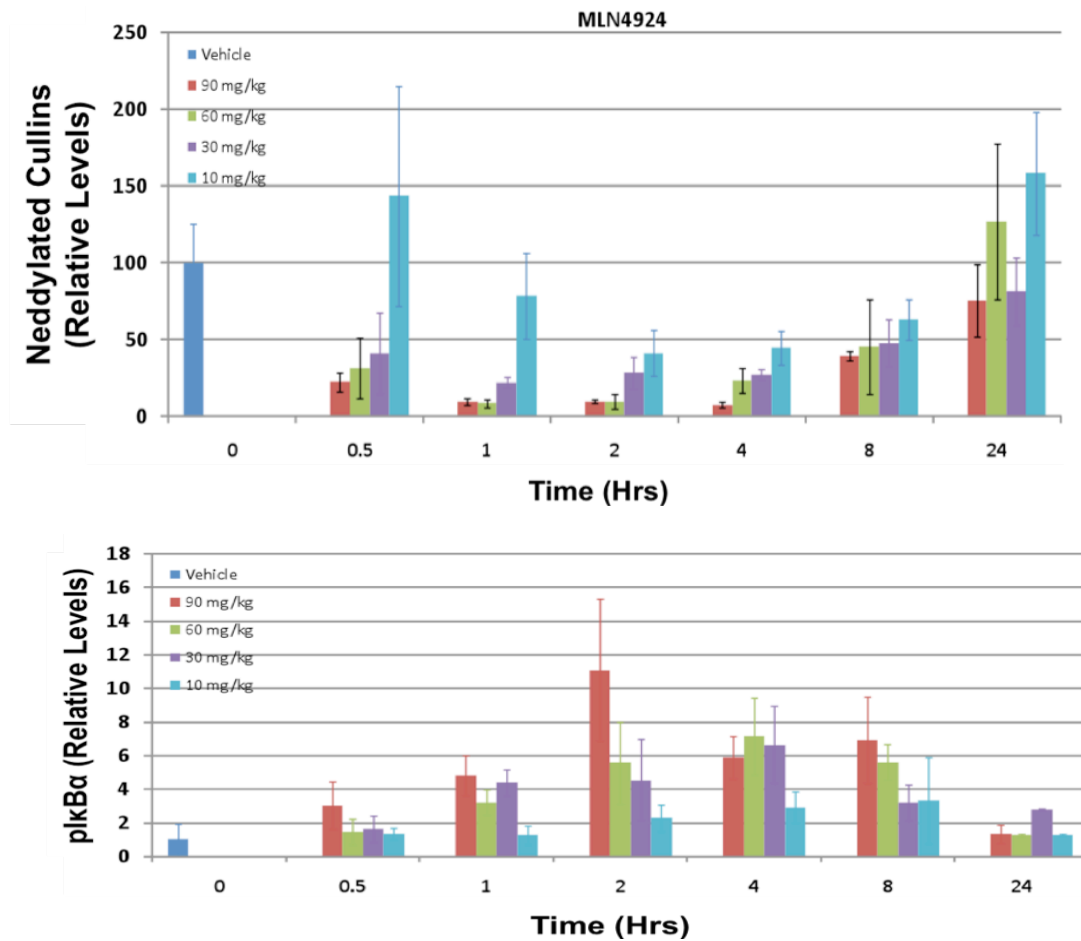


***Figure 2.5.12 Administration of MLN4924 to mice bearing HL-60 xenografts leads to a dose-dependent reduction in disease burden.***

Mice received vehicle control, 10, 30, 60, or 90 mg/kg MLN4924 BID for 21 days. Tumor volume was measured by calipers. Tumor regression was maximal for the higher doses and was durable for up to 70 days. The study was terminated close to day 100 due to tumor progression (data not shown).

#### 2.5.14 MLN4924 reduces neddylated cullins and stabilizes the cullin dependant substrate pIkB in vivo.

We wished to evaluate PD parameters in our animal experiment that would indicate pathway inhibition and potentially be useful in the clinic as biomarkers. We decided to analyze specimens collected from animals following administration of a single dose of MLN4924 and look for inhibition of cullin NEDDylation and accumulation of phospho-IkBa. MLN4924 reduced the levels of neddylated cullins in the tumor tissue analyzed and this event was predictably followed by stabilization of the cullin dependant substrate, pIkBa (figure 2.5.13).

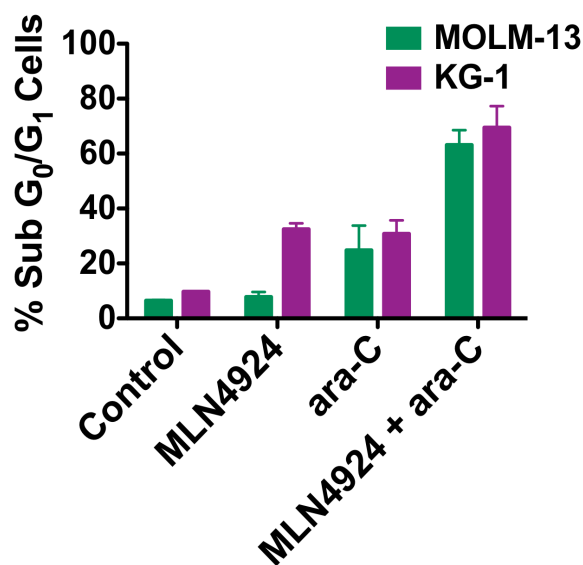


**Figure 2.5.13 Effect of MLN4924 treatment on NEDDylation of cullins in vivo.**

Mice were administered a single dose of MLN4924 and the levels of NEDDylated cullins were quantified at the indicated timepoints (top panel). Mice were given a single dose of MLN4924 and the levels of phospho-IkBa were quantified at the indicated timepoints post-drug administration (bottom panel).

**2.5.15 The cytotoxic effect of the standard agent cytarabine, is augmented by the addition of MLN4924 in vitro.**

Based on our collective data thus far, we felt that there was sufficient evidence to evaluate the activity of MLN4924 in the clinic as a single agent. However, we acknowledged the fact that approved agents in the clinic could potentially be augmented by the rational addition of new small molecule inhibitors and a potentially active combination would be worth evaluating in patients. Given the multifactorial mechanism of action of MLN4924, and the need to improve the activity of standard therapies, it was hypothesized that MLN4924 would augment the anti-leukemic effects of cytarabine. When combination experiments were conducted, MLN4924 dosed together with LDAC had at least additive activity when compared to the activity of either agent alone [117].

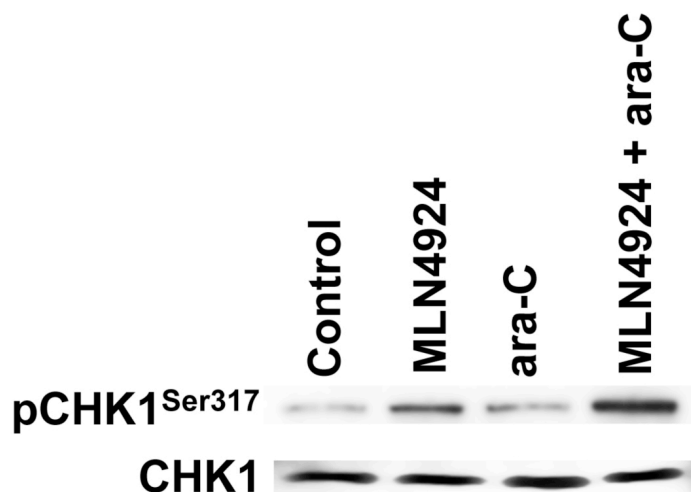


***Figure 2.5.14 Additive activity of MLN4924 combined with cytarabine***

MOLM-13 and KG-1 cells were treated with MLN4924 (100nM) and cytarabine (10nM) at the indicated concentrations both alone and in combination. Cells were incubated for 48 hours and sub-diploid DNA content was measured using PI/FACS analysis [117].

### 2.5.16 The combination of MLN4924 with cytarabine increases the expression of the DNA damage sensor CHK1

Given the DNA damaging effects of cytarabine, MOLM-13 cells were treated with both MLN4924 and cytarabine and induction of the DNA damage sensor CHK1, was measured. Greater pCHK1 induction was observed with the combination as compared to either agent alone [117].



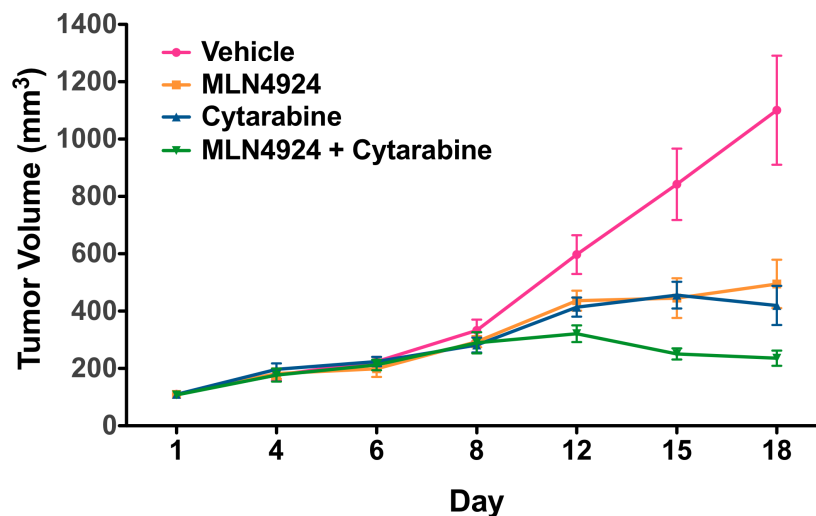
**Figure 2.5.15 DNA damaging effects of cytarabine is augmented with the addition of MLN4924.**

MOLM-13 cells were treated with MLN4924 (100nM), cytarabine (10nM) and the combination (for 24 hours). Protein lysates were prepared for western blot analysis and probed for the DNA damage sensor pCHK1 on its serine 317 residue. Equal loading was documented with total CHK1 levels [117].

### 2.5.17 MLN4924 combined with cytarabine is well tolerated in vivo and has greater anti-tumor activity than with either agent given alone.

Given the in vitro effects of the MLN4924/cytarabine combination outlined, the anti-tumor effect of this novel combination was evaluated in an vivo model. The activity of vehicle controls, MLN4924, cytarabine and the combination was studied in mice implanted with HL-60 xenografts. The MLN4924/cytarabine combination was well tolerated and led to a more potent tumor regression than with either agent given alone (figure 2.5.16).





**Figure 2.5.16 Administration of a cytarabine/MLN4924 combination to mice bearing HL-60 xenografts leads to a dose-dependent reduction in disease burden.**

Mice received vehicle control, MLN4924 (30mg/kg BID sc twice weekly x 2 weeks), cytarabine (75mg/kg ip three times weekly x 2 weeks), or the combination for 18 days. Tumor volume was measured by calipers [117].

## 2.6 Discussion

### 2.6.1 Introduction

Despite recent advances in the basic science understanding of AML, clinical progress with this disease has lagged behind significantly. Standard induction therapy for AML has remained fundamentally the same for the last 3 decades. This is reflected in recent SEER registry data, where the overall survival rate for AML between 2001 and 2007 in the United States was just 22.6%. In fact, the greatest advances in AML therapeutics have been in supportive care and improved stem cell transplantation methodologies. These improvements have translated into a modest survival benefit. Since there are only a few approved cytotoxic agents for the treatment of patients with AML, it is likely that outcomes will be dictated more by patient selection, rather than on the basis of

improvement in the biologic effects of currently approved agents, at least for the short term. It is clear that new therapies are needed for this disease and increasing accrual on clinical trials of novel agents is the only way that therapeutic progress can be made for these patients. Given this, we explored the role of NAE as a new target for drug development in AML. Several questions needed to be addressed in this project, for example, how reliant are AML blasts on NEDD8 protein conjugation for survival? Given the heterogeneity of this disease, would an NAE inhibitor have a selective effects in certain subgroups only? If activity was demonstrated, what mechanisms could underly this and would it be possible to develop rational drug combinations and biomarker strategies in the lab, for translation into the clinic?

### **2.6.2 MLN4924 – broad anti-tumor activity.**

We showed that MLN4924 had low nanomolar activity (IC<sub>50</sub>: 221nM) across a broad range of AML cell lines (**table 2.6.1**). Clearly, the number of patient samples assayed ex-vivo (n=5) are insufficient to confidently indicate that MLN4924 retains activity across good, intermediate and adverse risk AML. We expect to test more patient samples in the future to validate these findings

Cell Line	Provider	Source	Phenotype
HL-60	ATCC	Peripheral blood, 36 year old Caucasian female	Acute promyelocytic leukemia (APL)
MV-411	DSMZ	Peripheral blood, 10 year old Caucasian male	Biphenotypic B myeloid leukemia with FLT3 ITD
MOLM-13	DSMZ	Peripheral blood, 24 year old Caucasian male	Acute promyelocytic leukemia with FLT3 ITD
PL-21	DSMZ	Peripheral blood, 20 year old Caucasian male	AML (FAB 5a) with FLT3 ITD

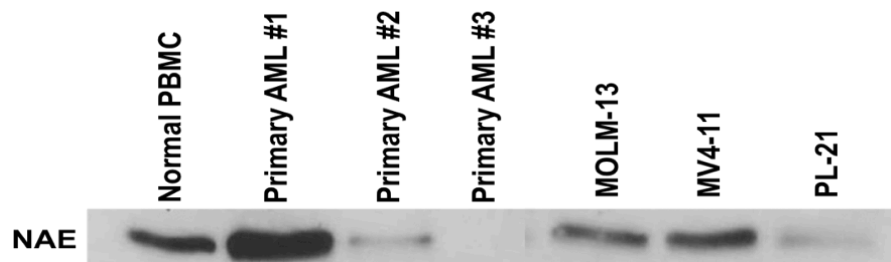
**Table 2.6.1 Cell lines used to test MLN4924 activity in vitro**

### **2.6.3 MLN4924 and drug resistance**

We did not explore the multi-drug resistance (MDR) status of the primary samples

collected, however, it would be interesting to show that MLN4924 activity was preserved despite documented MDR status. Importantly, is not clear at this time if MLN4924 is a drug efflux pump substrate. Pre-clinical studies have been set up to determine the pump expression of clinical samples (data not shown) using an IHC technique testing 2 MDR1 antibodies (C219 and JSB1). JSB1 appears to be quite specific and slightly less sensitive than C219. C219 gives a more robust signal but has cytoplasmic staining, suggesting that there may be some cross reactivity with a non-MDR1 antigen. We appreciate the limitations of interpreting IHC data in this context. The way the primary tissue is prepared leads to significant variation in results (type of fixative used, duration of fixation, method of preparing paraffin sections, time between cutting and staining etc). Flow techniques are probably more representative of expression and activity. Irrespective of the technique used, determining which pumps are of relevance is challenging and it should be noted that clinical efforts to modulate MDR status to improve the effects of standard agents have been largely unsuccessful [100-103].

In this study, we explored the activity of MLN4924 against other common mechanisms of drug resistance, namely, variable expression of the target (NAE), activation of Flt 3 signaling and the influence of bone marrow stromal cells. We showed wide variation in the expression of the target across a series of in-vitro and in-vivo samples (**figure 2.6.1**) . This observation however, did not impact on the potency of MLN4924.



**Figure 2.6.1** *Variable expression of NAE.*

Expression levels of NAE in PBMCs, cell lines and clinical samples were assayed by immunoblotting.

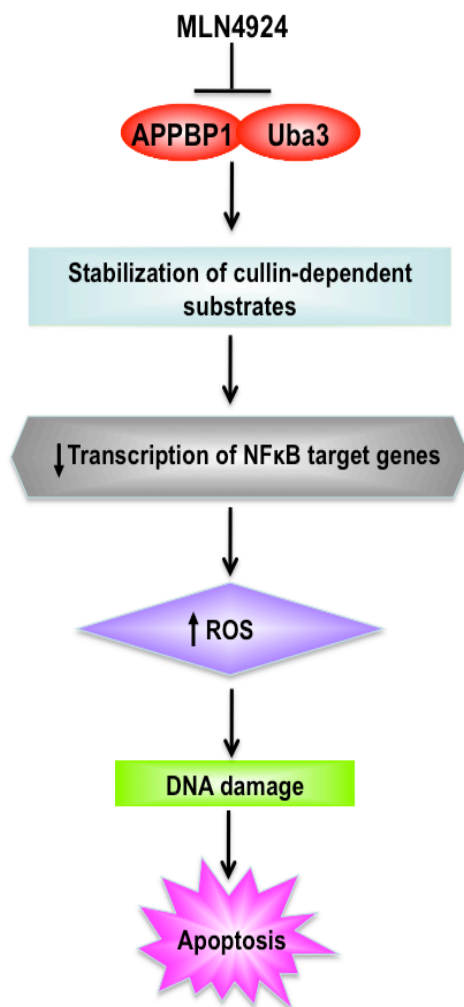
The expression of Flt 3 did not appear to modulate the activity of MLN4924 wild type and stably knocked down Flt 3 cell lines. In a co-culture system with H5-S bone marrow stromal cells MLN4924 could overcome the survival signals supplied by bone marrow stroma. It could be argued that the cell lines used were never reliant on stromal supportive signaling to begin with and to address this issue, we used a positive control where we showed that bone marrow stroma provided proliferative signals to HL-60 cells by increasing the number of viable cells measured over time. MLN4924 treatment in the same experiment overcame these survival signals (**figure 2.5.18**). To conclude, we failed to abrogate the activity of MLN4924 in vitro despite the generally accepted pre-clinical limitation of modeling in-vivo treatment resistance. We hope that these observations will have a positive influence on the clinical development of this agent.

#### **2.6.4 Selective Cytotoxicity of MLN4924**

In addition to having broad anti-leukemia efficacy we showed that MLN4924 activity was relatively selective for leukemic cells since we observed minimal effect on peripheral blood mononuclear cells at doses over 14 times higher than the IC<sub>50</sub> for cell lines. However, we do recognize the limitations of this assay and a more representative experiment to properly measure the effects of MLN4924 on normal human hematopoietic progenitors, would have been to look at the proliferation of CFU-GM, BFU-E and CFU-GEMM as assessed by colony assay on human bone marrow, cord blood or mobilized peripheral blood mononuclear cells. These experiments were not carried out, since as we will show in the next chapter, the clinical grade compound had almost no hematologic toxicity. In addition, others have shown that MLN4924 does not impair colony formation of normal hematopoietic stem cells and selectively disrupts the viability of leukemia stem cells and progenitor cells [104].

### **2.6.5 MLN4924 and oxidative damage**

Based on our mechanistic data, we proposed that through inhibition of NAE, the NFkB negative regulator I $\kappa$ B $\alpha$ , became rapidly stabilized. This event lead to reduced transcriptional activity of the NFkB p65 subunit which altered the expression of NFkB dependant target genes. Of these genes, we noted marked downregulation of the anti-oxidant protein, superoxide dismutase 2 (SOD 2). This led us to ask whether AML cells were being primed for apoptosis through increased production of reactive oxygen species (ROS). We went on to show that there was a marked increase in ROS production in AML cell lines treated with MLN4924 which ultimately led to apoptotic cell death in the treated cells. We noted that this effect could be blunted with the addition of the anti-oxidant enzyme n-acetylcysteine (NAC). We concluded that one of the principal mediators of MLN4924 activity was through the disruption of cellular redox status (**figure 2.6.2**).



**Figure 2.6.2 Schematic representation of MLN4924 mechanism of action**

Through disruption of NF-κB signaling, AML cells are primed for apoptotic cell death through ROS generation.

The increasing use of antioxidative dietary supplements in cancer patients has led to concerns about their potential impact on cancer cells and anti-cancer therapies [105]. Besides their ability to reduce treatment-related side effects [106] and increase the sensitivity of tumor cells to chemotherapeutic agents [107], antioxidative agents can also decrease the efficacy of cytotoxics by reducing their induction of oxidative stress [108]. Recent non-clinical studies indicate that the anti-oxidant vitamin C, abrogates the effects of the proteasome inhibitor bortezomib, either directly through physical interaction with

the drug, or indirectly through inhibition of the oxidative stress induced by impaired protein degradation [109]. Similar reports have appeared for epoxomicin and n-acetylcysteine (NAC). Given these observations, it would be interesting to elucidate the ROS generation that we observed in more detail since it could be argued that NAC might be physically interacting with MLN4924 to abrogate its activity. To understand the mechanism more thoroughly, over expressing SOD 2 or using an alternative anti-oxidant other than NAC to prevent ROS generation, would be helpful in ruling out potential physical interactions between NAC and MLN4924.

#### **2.6.6 MLN4924 and DNA damage**

Our data indicate that by inducing ROS generation, MLN4924 induces DNA damage and leads to apoptotic cell death. The role of ROS in inducing DNA damage would be strengthened if concomitant  $\gamma$ -H2AX foci had been shown to develop in cells as a marker of double stranded DNA breaks, especially if this remained the case in the presence of pan caspase inhibition (caspases can of course cleave DNA once activated). A kinetics experiment to confirm this mechanism would be informative. It should also be noted that while ROS generation appears to be a critical mediator of MLN4924 activity, the use of NAC did not completely abrogate its effects, implying that there may be other mechanisms contributing to MLN4924 induced cytotoxicity. Based on the DNA damaging effect of the approved agent cytarabine, it was hypothesized that MLN4924 might augment this effect. The combination induced greater CHK 1 induction than with either agent alone and led to at least additive loss of viability in cell lines. The exact mechanisms underlying this effect have not been fully explored.

#### **2.6.7 Conclusions**

In this study, we report for the first time that MLN4924, a first in class inhibitor of NAE, has potent activity in a pre-clinical model of AML. Collectively our data generates as many questions as answers, given the novelty of the pathway and the drug. We expect to answer these important questions in future studies. Importantly however, work done in our laboratory justifies the conduct of a phase I trial to explore the clinical activity of MLN4924 alone or combined with cytarabine, for patients with AML.

## **Chapter 3: A phase I dose escalation study of MLN4924, in adult patients with acute myeloid leukemia (AML) and high risk myelodysplastic syndromes (MDS).**

### **3.1 Introduction**

During our pre-clinical experiments with MLN4924, this agent was already being evaluated in the clinic with phase I clinical trials enrolling patients with advanced solid tumors (protocol C15001), lymphoma and myeloma (protocol C15002) and melanoma (protocol C15005).

#### **3.1.1 Protocol C15001**

This was a phase 1 study in adults with advanced non-hematologic malignancies evaluating the safety and tolerability of MLN4924 given daily for 5 days and repeated every 21 days. Dose escalation was based on an adaptive approach using the continual reassessment method (CRM). Dosing started at 25 mg/m<sup>2</sup> and proceeded to 83 mg/m<sup>2</sup>. Abnormal liver chemistries (AST, ALT and bilirubin) were observed during cycle 1 following both single and multiple doses. Transaminitis was found to be dose limiting at doses exceeding 50 mg/m<sup>2</sup>. Importantly, Grade 3/4 liver chemistries occurred in 2 patients, who both died during the first week of study treatment. One death occurred in a patient with widely metastatic breast cancer receiving 61 mg/m<sup>2</sup>, and the second occurred in a patient with advanced colon cancer receiving 83 mg/m<sup>2</sup>. In both cases, the investigators assessed the hepatic toxicity as possibly related to MLN4924. As a result of these deaths and their association with abnormal liver function, the MLN4924 clinical program was amended so that protocols included an eligibility criterion of normal bilirubin levels in addition to ALT/AST levels below twice the institutional ULN. The MTD for this schedule was reported as 50 mg/m<sup>2</sup> or 150 mg/m<sup>2</sup>/cycle.



Based on clinical observations from other studies suggesting intermittent dosing might be more effective and better tolerated, in addition to evidence of acute phase responses occurring in some patients (non-neutropenic fever, elevated CRP), 2 new schedules were opened [dosing on D 1, 3, and 5 every 21 days, with (schedule B) or without (schedule C) dexamethasone 8 mg prior to dosing on d 1, 3, and 5]. On schedules B and C, respectively, 17 and 16 pts were enrolled to 50 (n=12, 2), 67 (n=3, 10), or 89 (n=2, 4) mg/m<sup>2</sup>. Median age was 60.8 yrs; 58% were male. Diagnoses included colorectal cancer (CRC; 28%), melanoma (19%), and gastric cancer (13%). Pts on schedules B and C had a median of 3 and 1.5 cycles, respectively. Schedule B DLTs were elevated ALT at 50 mg/m<sup>2</sup> and hyperbilirubinemia at 67 and 89 mg/m<sup>2</sup>; schedule C DLTs were hyperbilirubinemia and AST elevation at 89 mg/m<sup>2</sup>. Schedule B and C MTDs were 50 and 67 mg/m<sup>2</sup>, respectively. Common AEs on schedule B were fatigue (47%), hyperbilirubinemia, elevated ALT, constipation, and anorexia (each 24%) and on schedule C were anemia, nausea (each 44%), hypocalcemia (38%), hypomagnesemia, myalgia, and hyponatremia (each 25%); grade  $\geq 3$  AEs were seen in 35% and 44% of pts, respectively. Six pts on schedule B (3 melanoma, 1 CRC, 1 head and neck, 1 SCLC) and 3 on schedule C (all CRC) had SD durable for  $\geq 4$  cycles. Preliminary MLN4924 PK profiles were similar on d 1 and 5, suggesting no apparent accumulation in plasma. Single-dose dex prior to MLN4924 did not appear to decrease MLN4924 plasma exposure. In cycle 1 MLN4924 was shown to exert predicted pharmacodynamic effects in tumor/non-tumor tissues (blood, skin), e.g. upregulation of CRL substrate Cdt-1 in tumor tissue, consistent with NAE inhibition. This study has now reached its accrual objectives and is expected to appear in the literature soon as a first in man study (paper submitted to Journal of Clinical Oncology).

### **3.1.2 Protocol C15002**

This phase 1 study in adults with lymphoma or multiple myeloma tested 3 dose schedules, each repeated on a 21-day cycle: Schedule A: Days 1,2, 8, and 9 (completed; MTD = 110 mg/m<sup>2</sup> or 440 mg/m<sup>2</sup>/cycle); Schedule B: Days 1,4, 8, and 11; and Schedule C: Days 1 and 8. Dose escalation was based on the continual reassessment method (CRM). The MTD for Schedule A (Days 1,2,8, and 9) was determined to be 110 mg/m<sup>2</sup>

(440 mg/m<sup>2</sup>/cycle) based on DLTs consisting of febrile neutropenia occurring at 65 mg/m<sup>2</sup> and muscle cramps (Grade 4) and myalgia (Grade 2 but intolerable) at 147 mg/m<sup>2</sup>. A total of 17 patients received 110 mg/m<sup>2</sup> including 3 patients during the escalation phase and 14 additional patients (7 with lymphoma and 7 with multiple myeloma) in an expansion phase. One patient in the 110 mg/m<sup>2</sup> expansion cohort experienced Grade 3 AST and ALT elevation, but this did not contribute to the definition of MTD. As of 10 June 2010, 7 patients have been enrolled on Schedule B; no DLTs have been observed on this schedule. Six patients have been enrolled on Schedule C; 1 DLT has occurred at 196 mg/m<sup>2</sup> (reversible Grade 3 renal failure). Testing of Schedule B continues at 196 mg/m<sup>2</sup> (784 mg/m<sup>2</sup>/cycle). In this study, MLN4924 displayed a multi-exponential pharmacokinetic (PK) profile with a half life of 4–9 hours, relatively low PK variability, and approximately dose-proportional increases in total plasma exposure. Pharmacodynamic evaluation confirmed target inhibition [96].

### **3.1.3 Protocol C15005**

In this phase I trial patients with metastatic melanoma and no symptomatic brain metastases received 1-hr IV infusions of MLN4924 on d 1, 4, 8, 11 (schedule A) or d 1, 8, 15 (schedule B) of 21-d cycles. Thus far, 19 patients have been enrolled to 7 dose levels on schedule A: 50 (n=2), 67 (n=2), 89 (n=2), 118 (n=4), 157 (n=2), 209 (n=5), and 278 (n=2) mg/m<sup>2</sup>. Median age was 60 yrs (range 34–73); 58% are male. DLTs comprised one episode of grade 3 hypophosphatemia (118 mg/m<sup>2</sup>) and one of grade 3 acute renal failure (278 mg/m<sup>2</sup>). The MTD has not been defined as yet. The most common AEs (any grade) included fatigue (58%), diarrhea (47%), nausea (42%), myalgia (37%), and vomiting (37%). Grade ≥3 AEs occurred in 5 pts, including the DLTs, cancer-related pain (n=2), and small bowel obstruction (n=1). One patient with BRAF-wild type tumor, with rapidly progressive disease at entry, achieved a PR at cycle 4 and continues on study. Nine (47%) patients exhibited SD, durable for 4 and 8 cycles in 2 patients. Preliminary PK data suggest no apparent accumulation of MLN4924 concentrations in plasma. Analyses of NAE-regulated transcripts in peripheral blood reveal the expected PD effects of MLN4924-target inhibition. Accrual continues at 209 mg/m<sup>2</sup> on schedule A; dose escalation from 157 mg/m<sup>2</sup> on schedule B is ongoing.

### **3.1.4 Protocol C15003**

Based on our pre-clinical evaluation of MLN4924 in an AML model together with the available clinical data in ongoing studies, we justified the conduct of a phase I clinical trial of MLN4924 for patients with advanced MDS and AML [50].

## **3.2 Project Aims**

**Aim 1** - To determine the safety profile and establish the MTD and recommended phase 2 dose of MLN4924 administered IV in patients with AML and advanced MDS.

**Aim 2** - To evaluate potential efficacy of MLN4924.

**Aim 3** - To describe the PK of IV-administered MLN4924 in plasma.

**Aim 4** - To investigate the pharmacodynamic (PD) effects of MLN4924 in peripheral blood.

## **3.3 Project Summary**

We treated 24 patients with advanced MDS and AML on protocol C15003 as part of a phase I clinical trial of MLN4924. In this study, MLN4924 was given as a one hour intravenous infusion on Days 1, 3 and 5 of a 21 day cycle. We reported 2 dose-limiting toxicities at 78 mg/m<sup>2</sup>. One patient got multi-organ failure in the context of sepsis (after 2 cycles), the other had reversible elevation of AST during cycle 1 which resolved after withdrawal from study. The most common adverse events (AEs) were pneumonia (n=6), atelectasis, constipation, diarrhea, and febrile neutropenia (each n=4); most common grade  $\geq 3$  AEs were febrile neutropenia (n=4), elevated AST, and pneumonia (each n=3). Four patients achieved a complete response (CR). A 29-year-old woman with relapsed AML following allogeneic stem cell transplantation achieved a CR after cycle 1 at 25 mg/m<sup>2</sup> before developing progressive disease at an extramedullary site during cycle 8. An 82-year-old male patient with high-risk MDS/AML who was unresponsive to azacitidine had a partial response in cycle 8 and a CR with incomplete recovery of blood counts

(CRi) in cycle 10 at 33 mg/m<sup>2</sup>; the patient was rendered transfusion independent after 12 cycles of treatment. A 71-year-old man with de-novo AML refractory to standard cytarabine plus daunorubicin induction achieved a CRi during cycle 1 at 44 mg/m<sup>2</sup>; although this was not maintained, the patient continued to benefit from treatment with reduced transfusion dependence. Pharmacodynamic data were available for 9 patients, 7 of these showed evidence of target inhibition in peripheral blood by changes in NAE-regulated proteins

### **3.4 Materials and Methods**

#### **3.4.1 Study Design**

This was an open-label phase I, non-randomized study for patients with refractory advanced MDS and AML. Starting doses were based on data from studies of MLN4924 in patients with refractory solid tumors, lymphoma/myeloma and metastatic melanoma. The primary objective of this trial was to determine the MTD for MLN4924 administered on days 1,3 and 5 of a 21 day cycle. The protocol and study documents were approved by the Institutional Review Boards of the 4 participating sites (at Stanford University, University of Michigan, Dana Farber Cancer Center and our site in San Antonio). The study procedures adhered to applicable regulatory requirements, Good Clinical Practice, and the Declaration of Helsinki. Written informed consent was obtained from all patients before any study specific procedure was performed.

#### **3.4.2 Patient Eligibility Criteria**

Patients with advanced myelodysplasia (bone marrow blasts exceeding 10%) and acute myeloid leukemia were eligible for this trial. Additional eligibility criteria included: age of at least 18 years; ECOG performance status of  $\leq 2$ ; adequate renal function (calculated creatinine clearance  $> 50$  mL/min); adequate liver function (bilirubin  $\leq$  institutional upper limit of normal, AST/ALT  $\leq 2.5$  times institutional upper limit of normal). Patients were excluded for: treatment with an anti-leukemic investigational agent (other than hydroxyurea) received within 14 days prior to entering study; B-type natriuretic peptide (BNP)  $> 1.5$  times institutional upper limit of normal; pregnancy or

breastfeeding; uncontrolled intercurrent illness and known infection with HIV and/or viral hepatitis B or C.

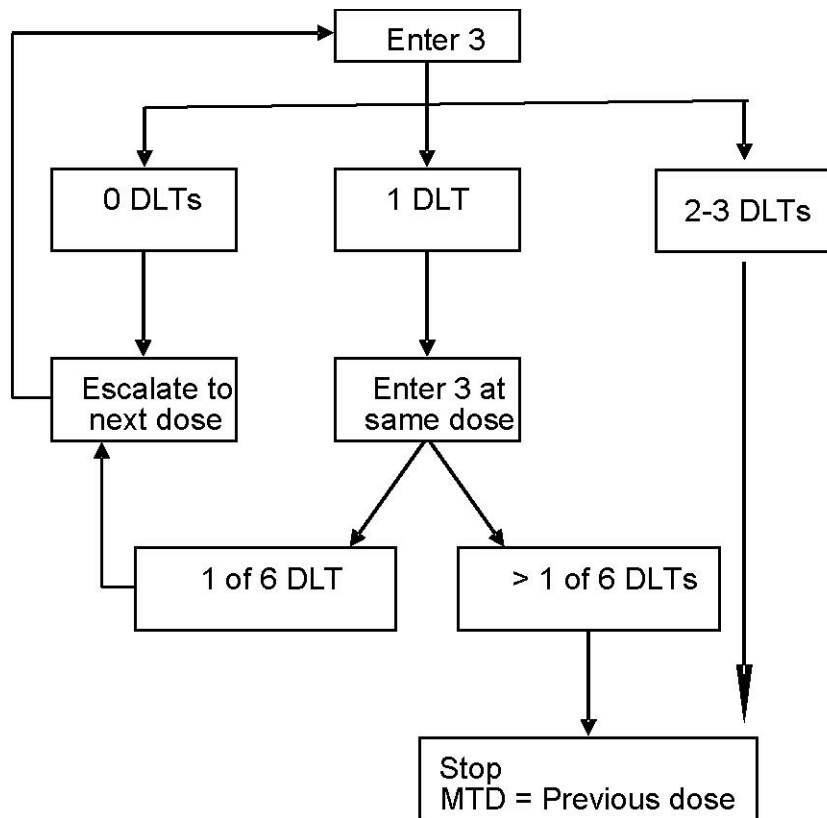
### **3.4.3 Study Assessments**

Patient demographics and medical history were recorded at baseline. AE, physical examination, vital signs and ECOG performance status were assessed at baseline and again weekly for the duration of the trial. Toxicity was graded using the National Cancer Institute Common Terminology Criteria for Adverse Events (version 3.0). Hematology, clinical chemistries and urinalysis were carried out on days 1, 3, 5, 8, and 15 of cycles 1 and 2. For cycles 3 and beyond, these laboratory investigations were collected on day 1 only. Patients remained on study until they no longer experienced clinical benefit, had progressive disease according to the investigator or experienced unacceptable toxicity. Patients were assessed for efficacy at the end of each 21-day cycle of therapy according to published standard response criteria.

### **3.4.4 Study treatment and dose escalation**

MLN4924 was administered as a 1 hour infusion on days 1, 3 and 5 of a 21 day cycle. Dose escalation was commenced at 25 mg/m<sup>2</sup> based on toxicity and response data available in other clinical studies of MLN4924. Dose escalation followed the standard “3+3” design (**figure 3.4.1**) and continued until the MTD was reached (**table 3.4.1**). More conservative dose escalation, evaluation of intermediate doses, and expansion of an existing dose level were all permissible following discussions between the investigators, if such measures were needed for patient safety or for a better understanding of the dose-related toxicity, exposure, or pharmacodynamics of MLN4924.

**Figure 3.4.1 – Dose escalation rules for MLN4924**



**Table 3.4.1 – Planned dose level dose level**

Dose Level	MLN4924 Dose
-1	15 mg/m <sup>2</sup>
1	25 mg/m <sup>2</sup>
2	33 mg/m <sup>2</sup>
3	Prior dose x 1.33

#### **3.4.5 Pharmacokinetic studies**

Blood samples for the determination of plasma MLN4924 concentrations were collected on cycle 1 of the study only. Samples were collected pre-dose, and again immediately, 30 minutes, 1, 2, 4, 6, 8 and 24 hours after completion of MLN4924 infusions. Additional samples were collected on days 3 (pre-dose) and 5 (pre-dose, immediately and 3-6 hours post dose). MLN4924 plasma concentrations were assayed by mass spectrometry.

#### **3.4.6 Pharmacodynamic studies**

Blood and marrow samples for the determination of MLN4924 tumor penetration and pathway markers were collected on cycle 1 of the study only. Cysteiny1-MLN4924 was injected into rabbits and antibodies were collected from plasma and developed for immunostaining. These anti-MLN4924 antibodies were used on clot preparations prepared from bone marrow aspirates collected at screening pre-dose, and again 3-6 hours after dosing on day 5. RT-PCR was used to analyze the expression of NAE-regulated proteins (Nrf-2, Nq-01, Scl-70) in whole blood and these samples were collected pre-dose on day 1, and again immediately, 1, 2, 4, 8 and 24 hours after completion of MLN4924 infusions. Additional blood samples were collected on days 5 (pre-dose and 3-6 hours post dose) and 8 (at the end of the scheduled visit).

### **3.5 Results**

#### **3.5.1 Patient characteristics**

We enrolled 24 patients on this clinical trial. The majority of patients were male (n=14, 58%) and caucasian (n=20, 83%). The median age of the patients was 60 years (youngest: 29 years old, oldest: 84 years old) and the majority had a performance status of 1 or less (n= 18, 79%). Most of the patients enrolled had a diagnosis of AML (n=22, 92%) with the majority of these having intermediate to poor risk cytogenetics (n=21, 95%). Diagnosis and PS data was not available on 1 patient (**table 3.5.1**).

**Table 3.5.1 MLN4924 patient demographics**

<b>Patient Characteristics</b>	<b>N=24</b>
<b>Median age, years (range)</b>	59.3 (29–84)
<b>Male, n (%)</b>	14 (58)
<b>Caucasian, n (%)</b>	20 (83)
<b>ECOG performance status, n (%)*</b>	
0	5 (21)
1	13 (54)
2	5 (21)
<b>Primary diagnosis, n (%)*</b>	
AML	22 (92)
MDS	1 (4)
<b>Median time since primary diagnosis, months (range)</b>	11.4 (1-147)
<b>Baseline cytogenetics, n (%)</b>	
Low risk	1 (4)
Intermediate risk	6 (25)
High risk	12 (50)
Not available	5 (20)
<b>FAB subtype, n (%)</b>	
M0	1 (4)
M1	4 (16)
M2	6 (25)
M4	1 (4)
M7	1 (4)
NA	11 (45)
<b>Prior therapies, n (%)</b>	
Stem cell transplantation	11 (46)
Cytarabine	7 (29)
Azacytadine	3 (12)
Daunorubicin	3 (12)
Decitabine	2 (8)
Etoposide	2 (8)
Gemtuzumab Ozagamicin	2 (8)
Idarubicin	2 (8)
Mitoxantrone	2 (8)
> 3 prior therapies	4 (16)

\* Primary diagnosis and ECOG performance status not available for one patient.



### 3.5.2 Treatment exposure

In this study, patients were treated across 5 dose levels before the MTD was reached. The median number of cycles received was 2 (range 1-12+), with 7 patients receiving  $\geq 4$  cycles and 3 patients receiving  $\geq 8$  cycles. Of the patients that were withdrawn from the study (n=13, 54%), the main reason this was progressive disease. Of the patients that came off study because of toxicity (n=4, 16%), 2 of these were the patients that experienced DLTs (table 3.5.2).

**Table 3.5.2 MLN4924 patient exposure**

<b>Dose Levels Received</b>	<b>Patients (n=24)</b>
25 mg/m <sup>2</sup>	3 (12%)
33 mg/m <sup>2</sup>	4 (16%)
44 mg/m <sup>2</sup>	3 (12%)
59 mg/m <sup>2</sup>	10 (42%)
78 mg/m <sup>2</sup>	4 (16%)
<b>Cycle Numbers Received</b>	
4 or more	7 (29%)
8 or more	3 (12%)
<b>Study Withdrawals</b>	
Progressive disease	8 (33%)
Adverse events	2 (8%)
Symptomatic deterioration	1 (4%)
Other	1 (4%)

### **3.5.3 Dose limiting toxicity (DLT) and maximum tolerated dose (MTD) determination**

Two DLTs were reported at  $78\text{mg/m}^2$  (dose level 5) in patients with AML. The first patient died from multi-organ failure and progressive disease in the context of sepsis with grade 3/4 elevations of liver transaminases. The second DLT observed was a grade 3 elevation of ALT. Given these toxicities, according to our study protocol, we selected dose level 4 ( $59\text{ mg/m}^2$ ) as the maximum tolerated dose and recommended phase II dose for this schedule of MLN4924 treatment.

### **3.5.4 MLN4924 adverse events**

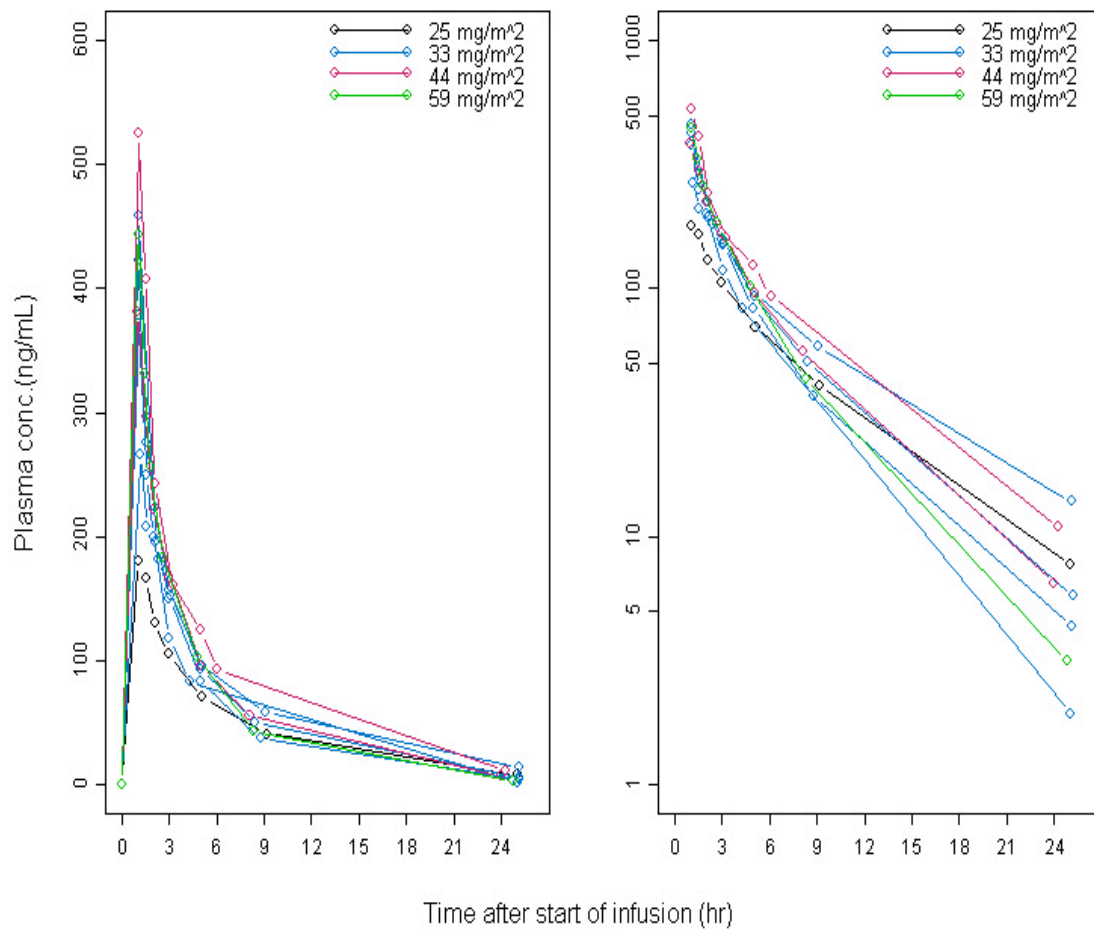
MLN4924 was well tolerated in general. The study protocol obliged us to report all adverse events occurring on study, however, not all the toxicities reported were directly attributable to MLN4924. There was a very low incidence of myelosuppression on study which appeared to be selective for megakaryopoiesis. Of the non-hematologic toxicities observed, those that were directly attributable to MLN4924 were pyrexia (which tended to be infusion related) and AST/ALT increases (which normalized between dosing). The patients that were discontinued as a result of toxicity were those that experienced DLTs.

### 3.5.4 MLN4924 toxicity data

<b>Adverse Event</b>	<b>All Grades, n (%)</b>	<b>Grade <math>\geq 3</math>, n (%)</b>
Any	21 (88)	17 (71)
<b>Hematologic toxicity</b>		
Thrombocytopenia	3 (13)	3 (13)
<b>Non-hematologic toxicity</b>		
Nausea	9 (38)	0
Diarrhea	8 (33)	0
Pyrexia	7 (29)	0
Chills	7 (29)	0
Febrile neutropenia	6 (25)	6 (25)
AST increased	6 (25)	4 (17)
ALT increased	6 (25)	1 (4)
Pneumonia	6 (25)	3 (13)
Abdominal pain	6 (25)	1 (4)
Dyspnea	6 (25)	1 (4)
Dizziness	6 (25)	0
Cough	6 (25)	0

### 3.5.5 MLN4924 pharmacokinetics

In 8 patients evaluable for PK, MLN4924 plasma levels peaked rapidly after dosing and then fell in a biphasic pattern. No accumulation of drug occurred between dosing. The estimated half life of MLN4924 was between 4 and 9 hours (**figure 3.5.1**).

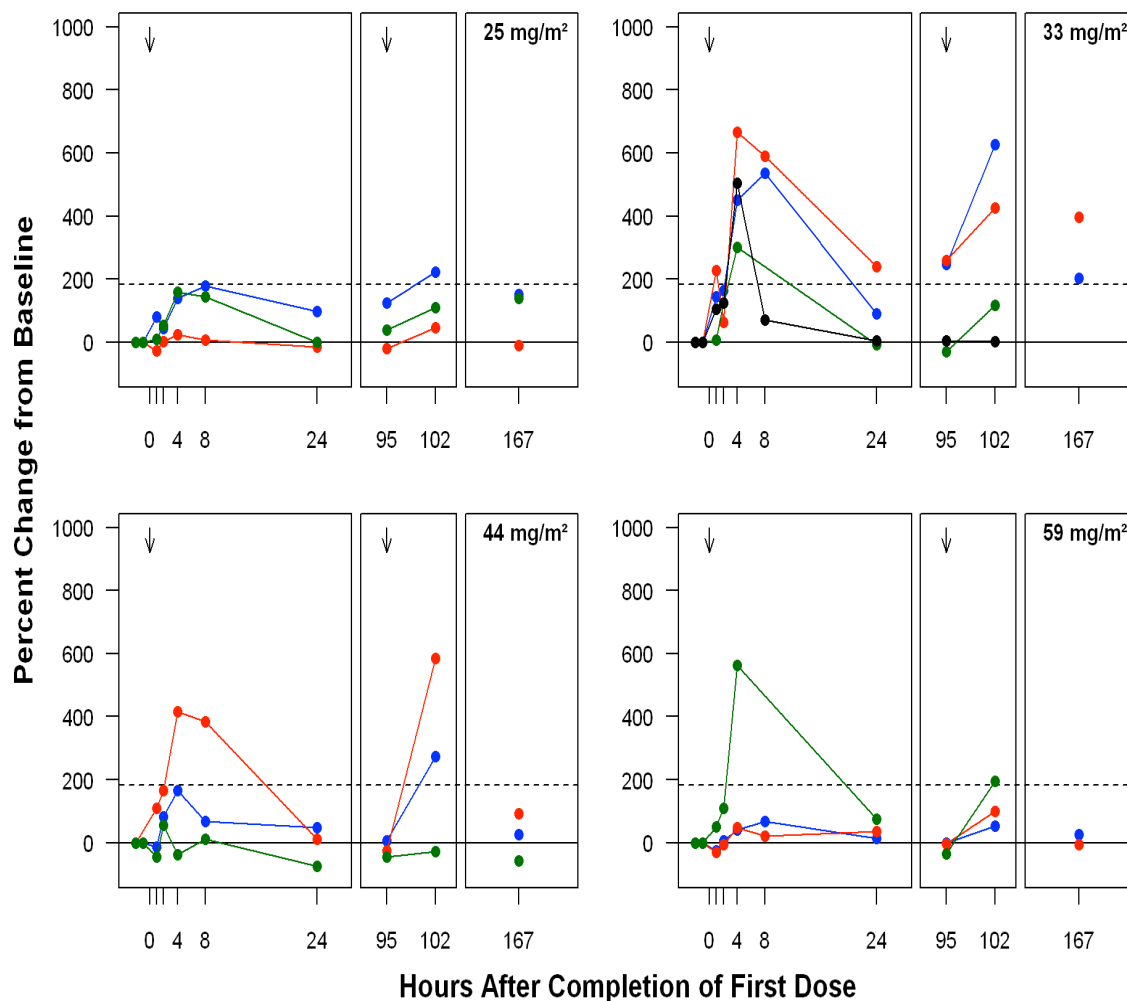


**Figure 3.5.1 MLN4924 pharmacokinetic data**

Plasma levels of MLN4924 were collected across 4 dose levels at pre-defined timepoints before and after dosing and plotted on linear and logarithmic scales. The estimated half life was 4-9- hours without formal compartmental modeling (thank you to Millennium Pharmaceuticals for providing help with this experiment).

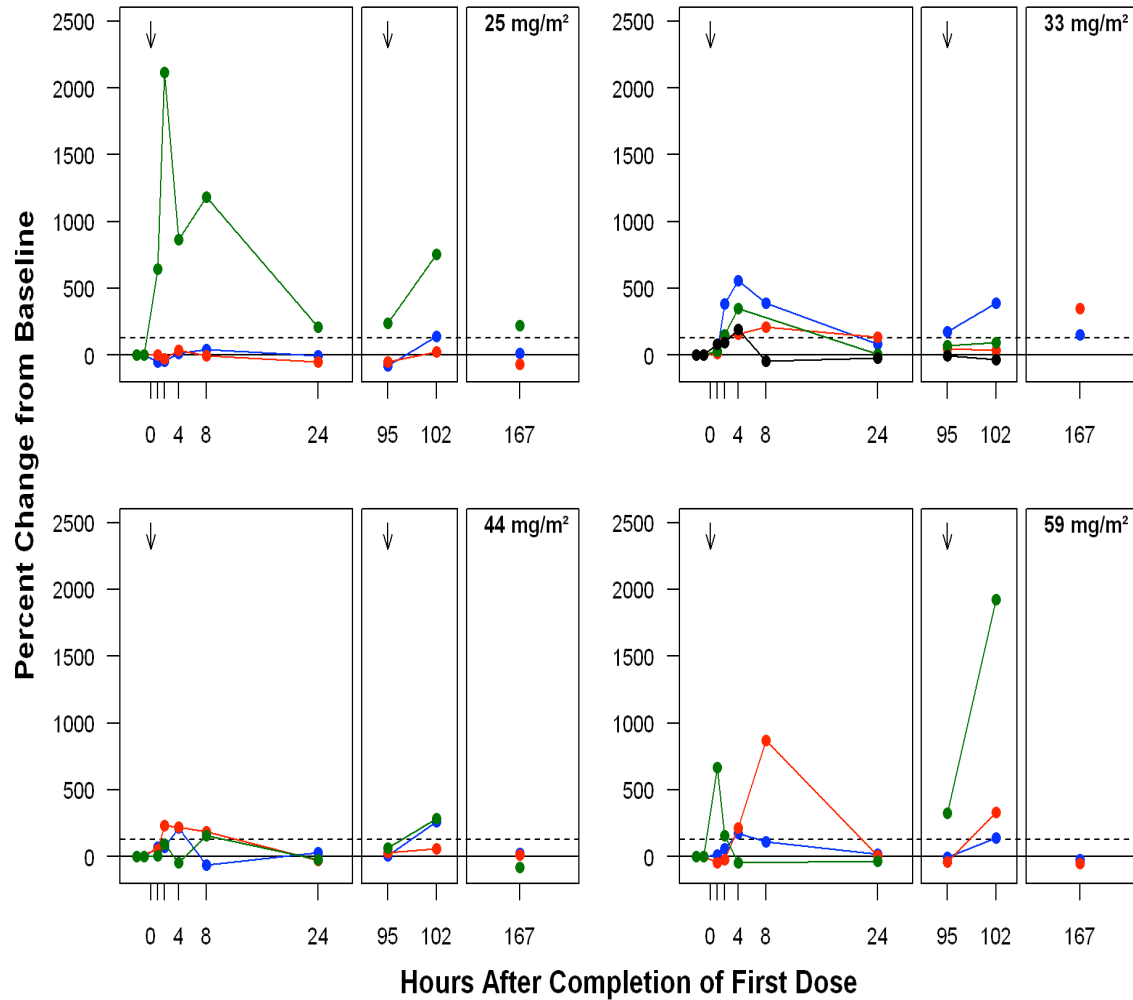
### **3.5.5 MLN4924 pharmacodynamics**

From our pre-clinical studies we showed accumulation of several cullin dependant substrates in MV-411 cells following treatment with MLN4924. One of these substrates was Nrf2, a transcription factor with a broad transcription program. We selected 2 Nrf2 dependant target genes as potential markers of MLN4924 pathway inhibition – NQO1 and SCL7A11. We measured both proteins by RT-PCR on days 1 and 5 (at pre-defined time-points) in the peripheral blood of 4 patients. We showed a dose dependant rise and fall of both protein transcripts indicating the expected consequences of NAE inhibition due to MLN4924 treatment (**figures 3.5.2 A & B**). In addition to assaying pathway markers in the peripheral blood, we were interested to investigate uptake of MLN4924 within leukemic blasts. We developed MLN4924 antibodies for immunohistochemistry as previously described and stained clot preparations collected from bone marrow aspirates pre-dosing on day 1 and again after dosing on day 5. We showed marked uptake of MLN4924 in tumor cells when we compared screening marrows with marrows collected after 3 infusions (**figure 3.5.3**).



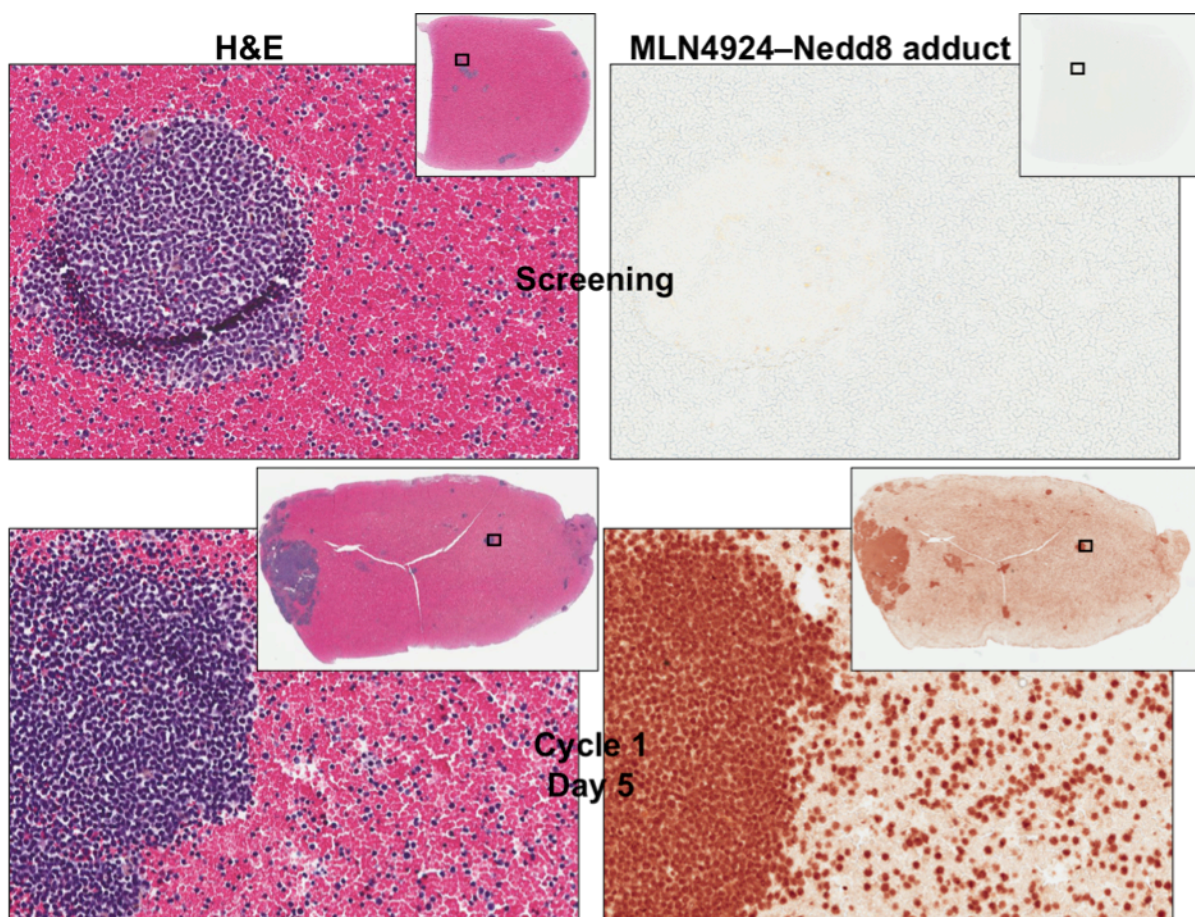
**Figure 3.5.2 (A) Percent change from baseline in NQO1 expression in blood following MLN4924 dosing at 25–59 mg/m<sup>2</sup>**

Blood was collected at predetermined time-points and NQO1 levels were measured by RT-PCR. Arrows indicate dosing on days 1 and 5. The patient represented by the blue line in the top left panel achieved a CR. No other responders were captured in this analysis (thank you to Millennium Pharmaceuticals for providing help with this experiment).



**Figure 3.5.3 (B) Percent change from baseline in SLC7A11 expression in blood following MLN4924 dosing at 25–59 mg/m<sup>2</sup>**

Blood was collected at predetermined time-points and SLC7A11 levels were measured by RT-PCR. Arrows indicate dosing on days 1 and 5. The patient represented by the green line in the top left panel achieved a CR. No other responders were captured in this analysis (thank you to Millennium Pharmaceuticals for providing help with this experiment).



**Figure 3.5.4 Immunohistochemical staining for MLN4924.**

Bone marrow clot preparations were prepared on day 1 (pre-dose) and 3-6 hours post dosing on day 5. Hematoxylin and eosin was used to identify the patients blast population on days 1 and 5 (left panel). Anti-MLN4924 antibodies were used on the same samples (right panel, thank you to Millennium Pharmaceuticals for providing help with this experiment).

### 3.5.6 MLN4924 clinical activity

We reported 4 CRs on this phase I trial (CR rate 16%, all 4 patients had AML, 1 had antecedent MDS) (**table 3.5.5**). The first patient to respond received the lowest dose level of MLN4924 (25 mg/m<sup>2</sup>). She was a 29-year-old woman with relapsed AML following allogeneic stem cell transplantation. This patient had a trisomy 8 abnormality



(+8[18]/46.XX[2]) and went on to get a complete cytogenetic remission of her disease which she maintained for 8 cycles of therapy before being taken off study because of relapsed disease. Patient #2 was an 82-year-old man with transfusion-dependent MDS/AML, which was unresponsive to prior therapy with azacitidine. This patient had a chromosome 20q12 deletion and received 33 mg/m<sup>2</sup> of MLN4924 and went to get a delayed response (morphologic CR) and transfusion independence before coming off study because of disease progression after 12 cycles of therapy. Two patients had CRs with incomplete count recoveries (CRi). The first was a 71-year-old man with de-novo AML refractory to standard cytarabine plus daunorubicin induction; He had a normal karyotype and was treated with 44 mg/m<sup>2</sup> of MLN4924 and received a CRi after his first course of treatment. He received a total of 18 cycles of therapy before coming off study because of gram negative sepsis. The second patient was a 51-year-old man with refractory AML, relapsing post allogeneic transplant that got a marrow CRi at the 59 mg/m<sup>2</sup> dose level.

**Table 3.5.5 MLN4924 responses.**

<b>Patients</b>	<b>MLN4924 dose (mg/m<sup>2</sup>)</b>					
	25 (n=3)	33 (n=4)	44 (n=3)	59 (n=10)	79 (n=4)	All (n=24)
<b>Morphologic CR</b>	0	1	0	0	0	1 (4%)
<b>Cytogenetic CR*</b>	1	0	0	0	0	1 (4%)
<b>CRi**</b>	0	0	1	1	0	2 (8%)

\* complete remission

\*\* complete remission with incomplete count recovery

## **3.6 Discussion**

### **3.6.1 Introduction**

Here we report for the first time the safety and tolerability profile of MLN4924 in patients with advanced myelodysplastic syndromes and acute myeloid leukemia. We showed that this agent could be safely administered with very little stem cell toxicity and infrequent drug related adverse events. We showed the consequences of NAE inhibition in peripheral blood and remarkable tumor penetration of MLN4924 into leukemic blasts from marrows collected before and after dosing. We also reported impressive clinical activity in patients with aggressive myeloid neoplasms. Importantly, our pre-clinical findings were successfully translated into the clinic for patients with major unmet needs.

### **3.6.2 MLN4924 toxicity profile**

Based on in-vivo toxicology studies conducted in dogs and rats the dose limiting toxicity in both species were GI toxicities (vomiting, diarrhea) and myelosuppression (pancytopenia). Other toxicities noted included acute phase responses with increased fibrinogen and decreased albumin. In dogs, neutrophilia was predominant, whereas in rats leukopenia was noted. Intravenous administration was well tolerated. Repeated subcutaneous injections at high concentrations and high doses used to assess toxicity in rats was associated with significant tissue injury characterized by necrosis and infection at the injection site. Thrombosis and myocardial degeneration were seen in the rats receiving higher doses subcutaneously but not in dogs or rats when administered IV. At the highest non-severely toxic doses (HNSTD) in dogs, slight prolongation in the activated partial thromboplastin time (aPTT) was noted. Electrolyte abnormalities noted in both species included decreased calcium, sodium, chloride, bicarbonate, and phosphorus.

Tissue sections revealed changes associated with the pharmacologic action of MLN4924 and included karyomegaly, mitotic figures and apoptosis in proliferating compartments of the GI, kidney, lymphoid organs, and skin in both rat and dog. In the dog, dose-related mild inflammatory changes characterized by scattered perivascular accumulation of

neutrophils occurred in a dose-related manner, and minimal to mild vasculitis and myocardial degeneration occurred at severely toxic doses in the rat.

The major toxicity of significance in this phase I trial was hepatotoxicity. Both DLTs were grade 3/4 elevations in liver enzymes. We decided that although drug attribution was possibly related, in the interests of safety, we attributed the death in the first patient that experienced a DLT to be directly related to MLN4924. This decision was taken because of a toxic death due to acute fulminant hepatitis that occurred on protocol C15001 where MLN4924 was being administered on days 1-5 every 21 days. This event led to a schedule change in that study, hence the day 1, 3 and 5 schedule employed for our study. The second DLT observed was a grade 3 elevation of ALT that occurred during cycle 2 of treatment. Although this fell outside the DLT period, because of the safety concerns outlined above, this event was considered a DLT. Importantly, this patient experienced normalization of liver function following withdrawal from study.

The exact mechanism explaining the hepatotoxicity observed is unclear. In-vitro, using RNA interference with a dominant negative Ubc12, (a NEDD8 E2 ligase), HEK293 cells (human embryonic kidney cells) and HeLa cell lines morphologically become swollen and edematous. This effect may relate to the accumulation of the cullin dependant GTPase RhoA, which regulates normal cytoskeleton function. The same morphologic abnormalities can be produced by treating cell lines with MLN4924, and reversed again by RhoA knockdown. The morphologic effects appear to vary and may be cell line specific. One possible explanation for the clinical toxicities observed, might be that same effect is occurring in the cytoskeleton of hepatocytes, and that there is leakage of transaminases because hepatocytes become swollen and enlarged. Since the liver tests revert to normal after withdrawing the drug it may be that this morphologic effect is temporary. Several other questions remained unanswered. For example, how important is protein neddylation for normal hepatocyte function when compared to other tissue types? Additionally, since we showed that MLN4924 disrupts cellular redox status as one of its principal mechanisms of action, how high is baseline ROS generation in normal hepatocytes and might these cells be more prone to NAE inhibition than those with lower

baseline levels? Regardless of the exact mechanism, patients with liver disease or who are taking concomitant CYP3A4 inhibitors (which MLN4924 relies on for clearance) need to be carefully monitored during the continued development of this agent.

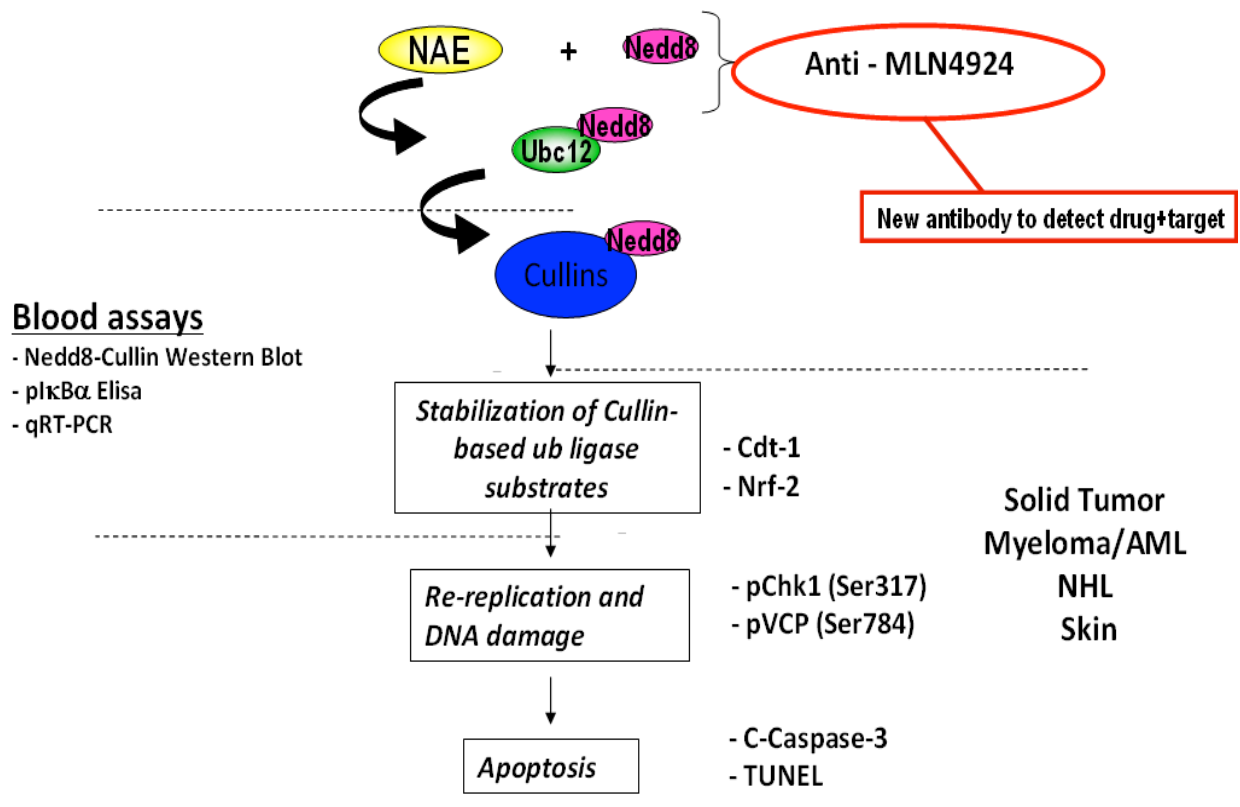
In general, MLN4924 was exceptionally well tolerated. Ten patients were treated at the MTD after the 2 DLTs were identified and no additional DLTs were reported at this dose level. As we predicted from our pre-clinical studies there was very little effect on normal stem cells with almost no myelosuppression observed. This makes MLN4924 an attractive molecule to combine with other established anti-leukemia drugs since additional myelosuppression is not likely to be dose limiting.

### **3.6.3 Pharmacodynamic activity of MLN4924**

Based on our previous pre-clinical data and the available clinical data, we had several opportunities in this phase I trial to explore biomarker strategies that would indicate the consequences of NAE inhibition and potentially identify sensitive patients in the clinic. Based on a detailed knowledge of the NEDD8 conjugation pathway, antibodies for IHC and western blotting in addition to RT-PCR assays were developed to detect changes in neddylated cullins, stabilization of cullin dependant substrates and the downstream events that resulted (activation of the DNA damage response, markers of apoptosis) (**figure 3.5.5**). Many of these techniques were already being optimized for ongoing studies in myeloma/lymphoma, solid tumors and melanoma. In our study, we collected PD samples at pre-defined timepoints to evaluate a selection of these pathway markers. Of these, we wanted to choose a biomarker that would reliably and reproducibly indicate that MLN4924 was interacting with its target. In this study, we showed that proteins regulated by the transcription factor Nrf-2 (which is a cullin dependant substrate which we showed previously to be stabilized following MLN4924 treatment in vitro), increased following MLN4924 treatment in vivo indicating pathway inhibition.

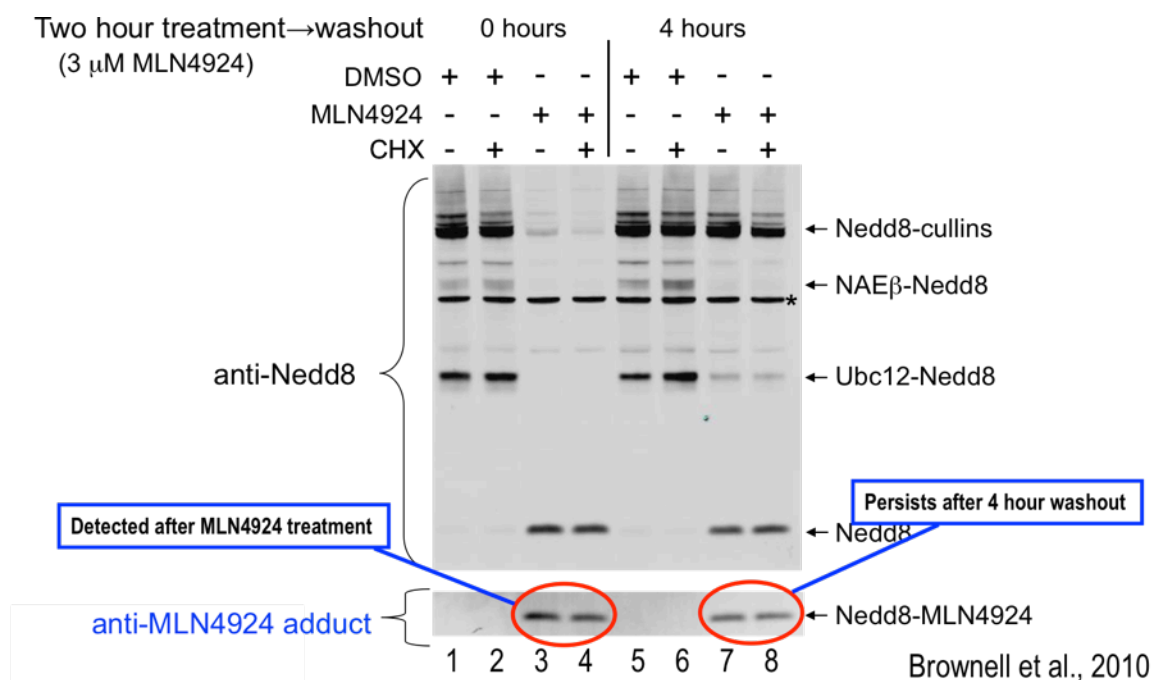
One of the particularly novel PD endpoints that we evaluated in this study was tumor penetration by MLN4924. Using antibodies developed from rabbit plasma we optimized an IHC technique to allow us to see uptake of MLN4924 in leukemic blasts from clot preparations made from bone marrow aspirates before and after drug treatment. In

addition, rabbit anti-MLN4924 antibodies were also developed for western blot analysis (**figure 3.5.6**). From xenograft studies in HL-60 mouse models, tumor tissue was collected serially following dosing and assayed for PD and PK endpoints. Proteolysis of MLN4924-NAE conjugates in the cell might account for the discrepancy between the PK signal (which decays after 24 hours) and the PD signal (which persists beyond 72 hours) although the exact reasons for the discrepancy observed are not clear (**figure 3.5.7**). Nonetheless, the anti-MLN4924 antibody technique was clearly a more reliable PD endpoint than an IHC technique for Nrf-2 (**figure 3.5.8**). It is worth noting that positive IHC stains for MLN4924 indicate that the drug is penetrating tumor tissue however, part of this signal will come from free drug that is not bound with its target. Development of antibodies that will specifically identify bound intracellular MLN4924 is currently underway. The MLN4924 biomarker strategy is part of an ongoing project, and although we can show that the drug produces a biologic effect in patients, we have yet to develop a biomarker that will allow us to identify potentially sensitive patients in the clinic.



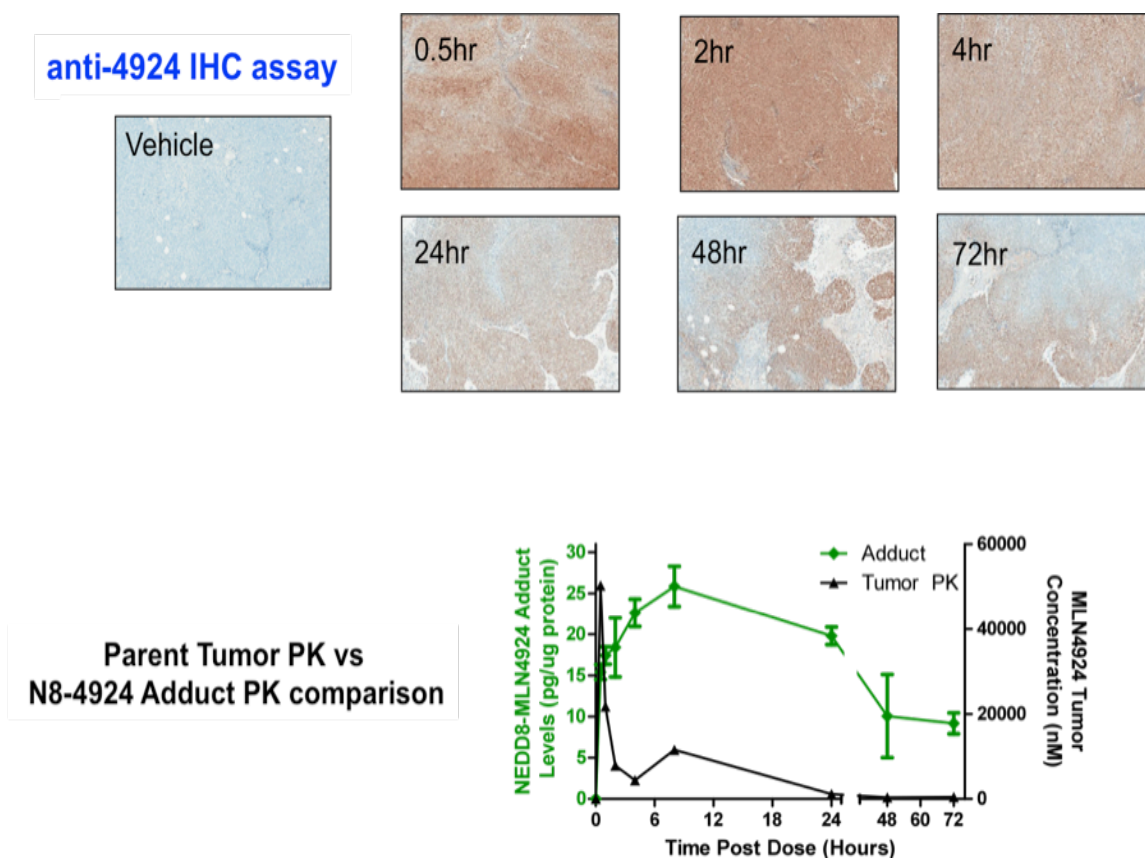
**Figure 3.5.5 – Pathway markers of NAE inhibition.**

Blood and marrow were collected at pre-defined time-points and several assays were carried out to develop a reliable biomarker strategy. Peripheral blood was collected and mononuclear cells were isolated for RT-PCR (for changes in protein transcripts regulated by cullin dependant transcription factors), ELISA (for plkB and others) and western blotting (to detect changes in E2 -NEDD8 conjugates and neddylated cullins). Bone marrow aspirates were collected at screening and again after 3 infusions of MLN4924 and clot preparations were stained by IHC for stabilization of cullin dependant substrates, (Cdt-1, Nrf-2), DNA damage (pChk-1), activation of apoptosis (cleaved caspase 3 and TUNEL assays) and tumor penetration by MLN4924 (using novel anti-MLN4924 antibodies developed for IHC).



**Figure 3.5.6 MLN4924 penetration in treated cells**

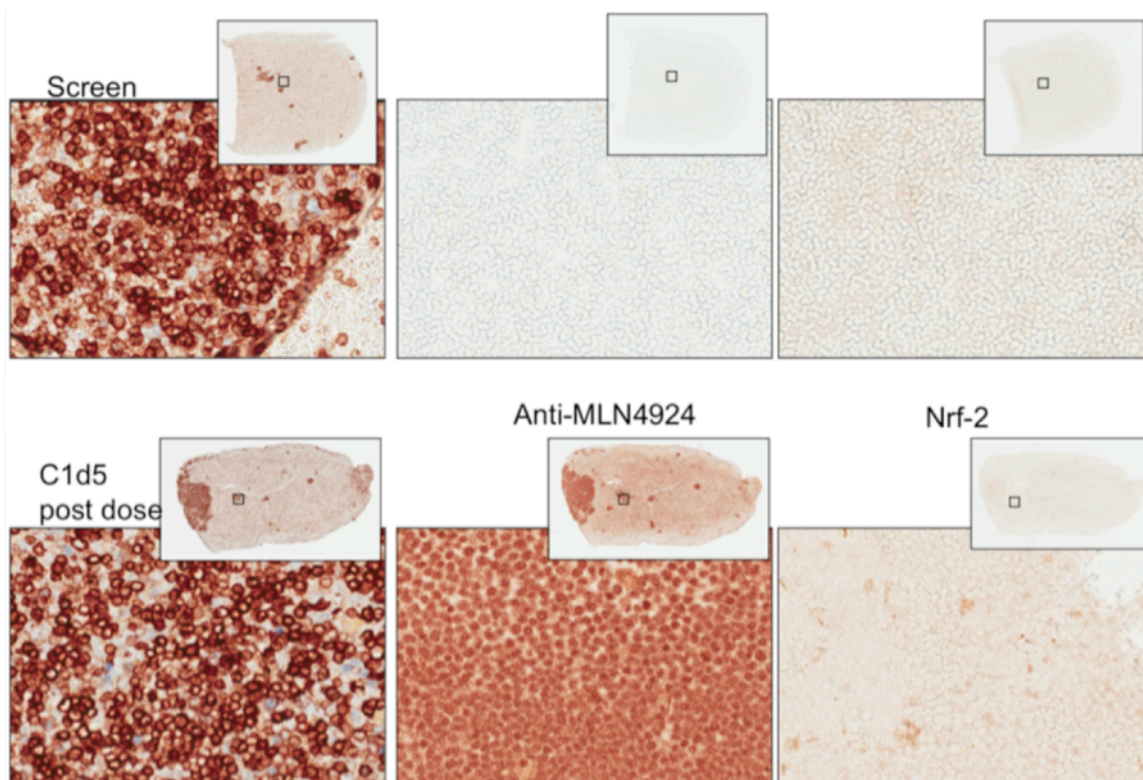
HCT116 cells were treated with 1 mM MLN4924 and harvested at the time points indicated. Cell lysates were processed for western blotting under nonreducing conditions and probed with anti-NEDD8, anti-MLN4924 antibodies and anti-E2-NEDD8 conjugates (Top immunoblot). HCT116 cells were treated with vehicle (lanes 1, 2, 5, and 6) or 3 mM MLN4924 (lanes 3, 4, 7, and 8) for 2 hr and then placed in fresh medium without inhibitor. Samples were removed at time 0 (lanes 1–4) or 4 hours (lanes 5–8) postwashout. Cells were also treated without (odd numbered lanes) or with 70 mM cycloheximide (CHX; even numbered lanes) throughout the experiment (bottom immunoblot) [47].



**Figure 3.5.7 MLN4924 pharmacodynamic and pharmacokinetic signals in a HL60 xenograft model**

HL60 cells were injected into the flanks of nude mice and allowed to grow to 150mm<sup>3</sup> being treated with MLN4924. Animals were then scarified and tumor tissue was collected at the indicated timepoints and then stained with anti-MLN4924 antibodies (top panel). Persistence of MLN4924 by IHC (pg/microg protein) and by quantification of tumor drug levels (nM) were recorded and plotted against time (hours) following dosing (bottom panel, thank you to Millennium Pharmaceuticals for providing this data).





**Figure 3.5.8 – Comparison of MLN4924 and Nrf-2 antibodies as biomarkers**

Bone marrow aspirates were obtained from treated patients and clot preparations were made at screening and after dosing on C1 D5. Myeloblasts were identified by CD34/CD117 co-stains (far left). Tumor tissue was stained with the indicated antibodies at the indicated time points (thank you to Millennium Pharmaceuticals for providing this data).

### 3.6.4 Pharmacokinetic activity of MLN4924

In this study, we showed a rapid increase in MLN4924 plasma levels with a biphasic decline when concentrations were plotted on a linear curve. Without having carried out formal compartmental modeling studies, we predict that a 2-compartment model will be sufficient to accurately assess the half life of this agent (which is probably somewhere in the region of 4-9 hours). Importantly, no drug accumulation occurred between dosing and all 8 patients evaluable for PK analysis achieved plasma concentrations of MLN4924 that exceeded concentrations known to inhibit NAE activity. Irrespective of this observation, MLN4924 given in doses of 25mg/m<sup>2</sup> as part of the current schedule, is clearly sufficient to inhibit the target given the CR that we reported at this dose level.

#### **3.6.4 MLN4924 – first in class activity**

We reported an overall CR rate of 16% on this study (2 CRs, 2 CRis). Patient #1 was the third patient to enroll on study and received the lowest dose level of MLN4924 (25 mg/m<sup>2</sup>). She was a 29-year-old woman with relapsed AML following allogeneic stem cell transplantation. This patient had a trisomy 8 abnormality (+8[18]/46.XX[2]) and went on to get a complete cytogenetic remission of her disease which she maintained for 8 cycles of therapy before being taken off study because of relapsed disease. Patient #2 was an 82-year-old man with transfusion-dependent MDS/AML, which was unresponsive to prior therapy with azacitidine. This patient had a chromosome 20q12 deletion and received 33 mg/m<sup>2</sup> of MLN4924 and went to get a delayed response (morphologic CR) and transfusion independence before coming off study because of disease progression after 12 cycles of therapy. Two patients had CRs with incomplete count recoveries (CRi). The first was a 71-year-old man with de-novo AML refractory to standard cytarabine plus daunorubicin induction; He had a normal karyotype and was treated with 44 mg/m<sup>2</sup> of MLN4924 and received a CRi after his first course of treatment. He received a total of 18 cycles of therapy before coming off study because of gram negative sepsis. The second patient was a 51-year-old man with refractory AML, relapsing post allogeneic transplant that got a marrow CRi at the 59 mg/m<sup>2</sup> dose level. The responses post transplant observed are noteworthy. It would be interesting to evaluate changes in transplant associate antigens which might prime patients for a more potent graft vs leukemia effect if these anitgens were regulated by NEDD8 conjugation (however, neither of the 2 patients that responded post-transplant experienced clinical GVHD). Studies of MLN4924 in GVHD mouse models are now ongoing to explore potential immunomodulatory activity of this agent. Since only 4 responders have been reported so far, it is difficult to identify any commonality that might indicate a potentially sensitive patient population but we hope, that as more responders appear on study, that useful leads will emerge to explain the mechanism of action of this new agent in more detail.

## **Chapter 4: Future perspectives and conclusions**

### **4.1 Future Perspectives**

#### **4.1.1 MLN4924 – current status in the clinic**

As experience with MLN4924 in the laboratory and in the clinic mounted, several changes were introduced to protocol C15003 to incorporate new knowledge and optimize the study design. These changes principally involved:

1. The introduction of 3 new dose schedules and the addition of patients with ALL onto the study.
2. Collection of PD samples to explore mechanisms of drug resistance.
3. Precluding the use of statins, based on a safety analysis of the entire MLN4924 program.

#### *1. New Dose Schedules*

In parallel with protocol C15003, protocol C15002 was enrolling myeloma and lymphoma patients into 3 different 21-day cycle schedules. Schedule A patients received MLN4924 on days 1,2,8 and 9. On this schedule the MTD was established at 110mg/m<sup>2</sup> and myalgia was dose limiting. On schedule B, patients were dosed on days 1,4,8 and 11 (on a bortezomib type schedule). No DLTs were reported at 147mg/m<sup>2</sup> and dose escalation is ongoing. On schedule C, patients were treated on days 1 and 8 and again, no DLTs were seen at 147mg/m<sup>2</sup>. In addition, non-clinical PK/efficacy modeling based on murine xenograft experiments indicated that MLN4924 efficacy was related to total drug exposure in a schedule-independent manner. Across 5 different schedules over 3 different degrees of drug exposure (as measured by AUC) efficacy clustered by drug exposure, regardless of schedule (data not shown). Therefore, based on the clinical safety experience gained from protocol C15002 and the pre-clinical PK data outlined, 2 new dose schedules were added to protocol C15003. Schedules B and C dosed patients on days 1,4,8, and 11 and days 1,8 and 15 respectively. The intention was to allow further dose escalation by reducing the frequency of dose limiting hepatic toxicity, and at the

same time, deliver a higher exposure of drug to per cycle. Considering that relatively low doses of MLN4924 were already shown to have anti-leukemia activity, the proposed changes were introduced to increase the likelihood of capturing more responders. On schedules B and C, patients were escalated from higher baseline dose levels because of the available safety data on protocol C15002 so that higher doses would maximize anti-tumor activity per cycle and reduce the numbers of patients on less active doses.

Following the introduction of these amendments, the intention was to establish MTDs for schedules B and C and to enrich at those dose levels so that there would be a total of 9 patients with AML or MDS and 9 patients with ALL in each expansion cohort. Patients with ALL were added based on the observation that MLN4924 activity might be in part dependant on the cell cycle (based on available pre-clinical and clinical data, the activity of MLN4924 appeared to be greatest in tumors with higher proliferative fractions eg AML and diffuse large B-cell lymphomas). As the protocol progressed, it was decided based on accrual, toxicity and response data, that schedule B would be expanded only, with separate cohorts for both AML and advanced MDS, and an additional cohort for low grade MDS (which would replace the ALL cohort). The study is now currently accruing patients on schedule B so that once the MTD for this schedule has been established, additional patients will be enrolled at this schedules MTD for a 48-patient expansion cohort (16 patients with low-grade MDS, 16 with high grade MDS and 16 with AML).

Finally, based on pre-clinical synergy data and plans to explore a randomized study of MLN4924 given with and without azacytadine, a third dose schedule was introduced. This schedule added azacytadine in standard fixed doses combined with escalating doses of MLN4924 as per schedule B. This dose schedule is currently enrolling.

## *2. Resistance testing*

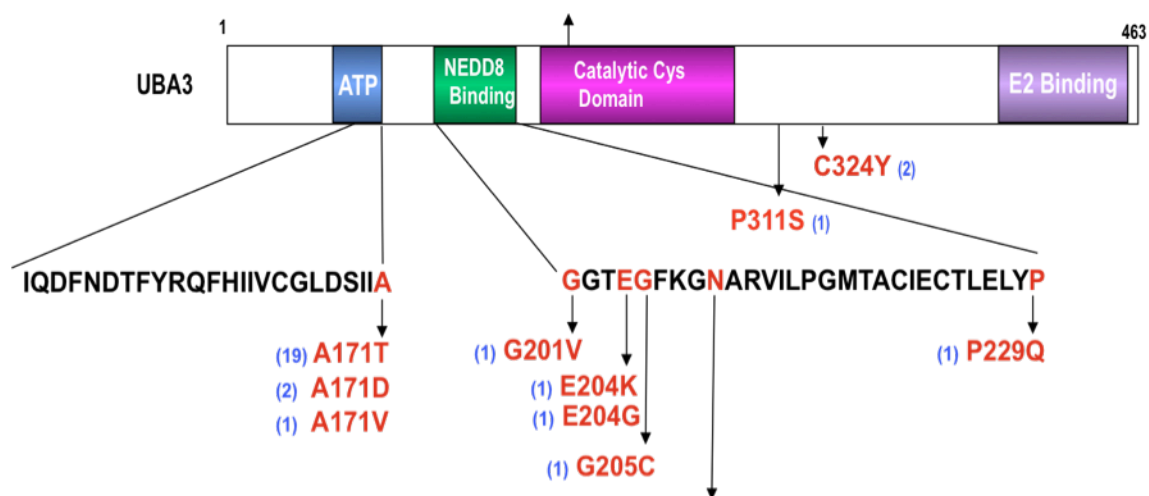
Based on the observation that all patients achieving CRs on study subsequently went on to progress, the possibility of treatment emergent mutations occurring that allowed escape from sensitivity to NAE inhibition was explored. This of course is one of the principal mechanisms for disease progression in chronic myeloid leukemia (CML) patients that

lose responses when treated with abl-kinase inhibitors. Following on from this experience in the clinic, in vitro and in vivo studies were conducted to identify target mutations that might have clinical relevance for patients treated with MLN4924.

To characterize potential mechanisms of resistance to MLN4924, xenograft models of AML were used [110]. Mice bearing HL-60 xenografts were treated with MLN4924 twice weekly for 110 days. Four of ten of these mice became treatment refractory and re-grew their tumors during the MLN4924 treatment period. These tumors were harvested and subjected to microarray analysis and DNA sequencing of genes required for NEDD8 activation by NAE. The enzymatic activity of NAE is delivered by two protein subunits, NAE- $\alpha$  and NAE- $\beta$  (UBA3). Mutations in NAE $\beta$  resulting in amino acid substitutions were detected in several xenografts in both the ATP binding pocket (A171T) and NEDD8-binding cleft (E204K) suggesting that mutations in the target enzyme may play a role in emergence of refractory tumors. Tumors containing mutations in NAE $\beta$  were passaged into new mice and shown to be completely insensitive to MLN4924 confirming resistance. In addition, cell lines derived from the resistant xenografts were shown to be resistant to MLN4924 in vitro but remained sensitive to other chemotherapeutic agents. Biochemical analysis of NAE $\beta$  mutants revealed a slower rate of NEDD8-MLN4924 adduct formation and demonstrated that the adduct was no longer bound tightly by the mutant enzyme. Thus, treatment emergent mutations in NAE $\beta$  lead to MLN4924 resistance in pre-clinical models through reduced inhibitory potency. These data serve as the framework for understanding mechanisms of resistance to MLN4924, provide rationale for patient selection approaches and offer a strategy to overcome treatment emergent mutations that may arise in clinical studies. Therefore, based on these data, blood and marrow collections were added at screening, during treatment, and at progression, to explore the possibility that UBA3 mutations (in particular the A171 mutation) might predict for response/progression in AML patients getting MLN4924 (**figure 4.1.1 A & B**).

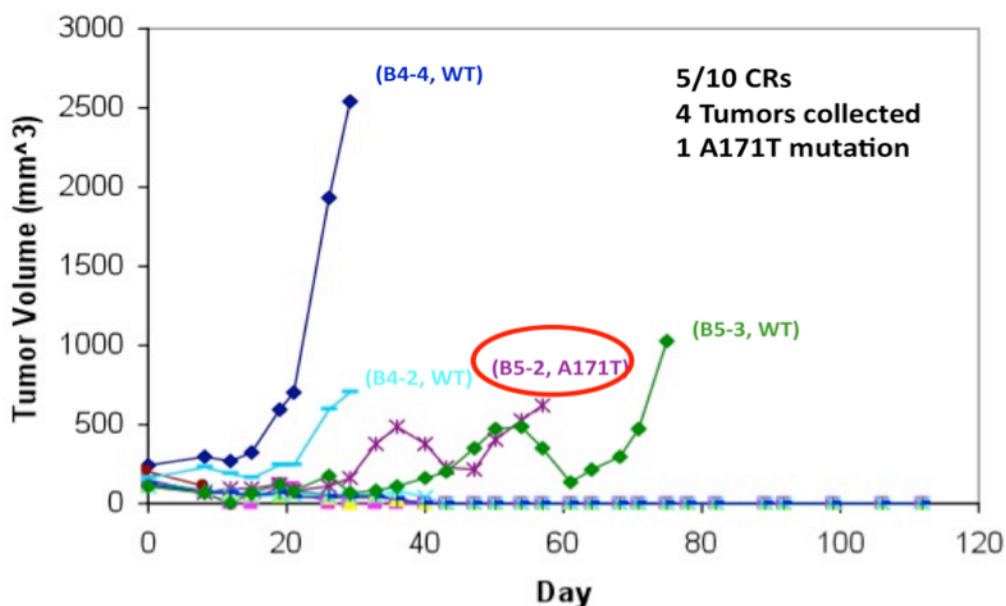
### 3. Safety amendments

As of January 2011, 140 patients had been treated with MLN4924 across 4 separate trials. Seven SAEs were reported describing renal insufficiency. In 5 of these 7 cases, the patients were taking statin like drugs (simvastatin for 3 patients, atorvastatin for 1 patient and lovastatin for 1 patient) at the time of enrollment. Approximately 15% (21 of 140) of all the MLN4924 treated patients were taking statins. This observation raised the possibility that patients on statins may be at greater risk for renal events. Patients taking statins as a result were required to come off treatment prior to, on the day of, and on the day after treatment with MLN4924. Based on these data, patients were also encouraged to pre-hydrate before coming on protocol and to avoid nephrotoxins during the trial period.



**Figure 4.1.1 (A) Heterozygous mutations detected in two regions of UBA3**

The main coding regions of UBA3 were sequenced as shown to reveal a nucleotide binding domain, a NEDD8 binding domain, a catalytic domain and an E2 binding domain. Several mutations were identified however, the A171 site within the ATP binding domain appeared as a “hotspot”, appearing in 22/31 mutations detected at this site from in vitro and in vivo studies.



**Figure 4.1.1 (B) A171 mutations in murine models**

A171 mutations were validated in pre-clinical models as recurring mutations potentially mediating clinical resistance. Of 10 mice treated with MLN4924 (180mg/kg BID/BIW) in a HL60 xenograft study, there were 5 of 10 tumor responses. Tumors were collected in the mice with progressing disease and 1 A171T mutation was detected in 4 tumors collected.

#### 4.1.2 MLN4924 – planned studies

Based on data reporting increased cytotoxicity from cytarabine when combined with MLN4924, we developed a protocol to evaluate the combination of low dose cytarabine given with escalating doses of MLN4924 in the clinic for patients with AML and advanced MDS. The primary objective of this trial will be to establish the safety and tolerability of the combination. Secondary objectives will include analysis of pharmacokinetic and pharmacodynamic endpoints as well as a preliminary description of anti-tumor activity. Although pre-clinical data suggests that the combination of MLN4924 and LDAC is at least additive, we plan to formally calculate a combination index (CI) using software based on the Chou-Talalay model (CalcuSyn). This will objectively confirm whether the combination is synergistic, additive or antagonistic (unlikely). In this study we also plan to determine the fold change in the levels of

phospho-CHEK1 induced by treatment with MLN4924/LDAC and to investigate the pharmacodynamic change in the expression of key genes following treatment with MLN4924/LDAC by FACS analysis and qRT-PCR. We plan to conduct a preliminary assessment of a potential correlation between the fold change in the levels of pharmacodynamic markers and the response to treatment if the data permit. This study has received local IRB approval (An open-label dose escalation phase 1 study of MLN4924, a novel inhibitor of Nedd8 activating enzyme, given in combination with low dose cytarabine in adult patients with acute myelogenous leukemia and advanced myelodysplastic syndromes – IDD #11-30) and is fully funded by research support from Millennium pharmaceuticals (Grant #X15002, award \$205,223). The project is expected to get underway toward the end of 2013.

The combination of azacitidine and MLN4924 has been investigated in non-clinical studies [111]. Both agents were shown to result in significantly increased DNA-damage and cell death compared to single agent alone as measured by western blotting and FACS analysis of cell cycle distributions. In vivo studies were performed in HL-60 and THP-1 xenografts using MLN4924 on a clinically relevant dosing schedule twice weekly. Single agent azacitidine at its Maximum Tolerated Dose (MTD) had minimal activity in the HL-60 model and was given with a sub-optimal dose of MLN4924 that when combined induced complete and sustained tumor regressions. The mechanism for the apparent synthetic lethality in this in vivo model has not been fully evaluated, however it is supported by a dramatic elevation in DNA damage and cleavage of caspase-3 in vivo in the combination arm. A second xenograft model (THP-1) that was also insensitive to single agent azacitidine treatment underwent complete and sustained tumor regressions when combined with MLN4924. Given the non-overlapping toxicity profiles of both agents and the clinical activity for each agent previously reported [112-114], a large phase III trial is planned. Based on safety data emerging from C15003 evaluating the MLN4924/azacitidine combination outlined above, it is expected that approximately 670 AML and advanced MDS patients will be randomized from 150 study centers in North and South America, Europe and Asia. This trial is expected to open in the summer of 2012.



## 4.2 Conclusions – a bench to bedside story

This thesis attempts to follow the fundamental process of translational research where an idea that is carefully developed in the laboratory, is then successfully carried into the clinic. The Nedd8 conjugation pathway is a relatively new pathway in cancer therapeutics. The NAE inhibitor MLN4924, is a novel small molecule that selectively disrupts this pathway and causes cancer cell death in acute myeloid leukemia. Several questions relating to the pathway and to the drug had previously been unanswered. Others had shown that the antitumor activity of MLN4924 was linked to two principal mechanisms in pre-clinical models: (1) the induction of DNA re-replication and cell death through dysregulation of Cdt-1 and (2) the inhibition of NF- $\kappa$ B signaling [47-49, 57, 115]. However, no studies were ever conducted exploring MLN4924 activity in AML. What were the mechanisms underpinning anti-tumor activity in this disease? Would the drug be well tolerated in the clinic? What dosing schedule might we test in the clinic for maximum therapeutic benefit and minimum toxicity? Could we use pharmacodynamic biomarkers to determine whether the compound blocked its target in patients in the same way it did in the laboratory (proof of mechanism)? What effect might modulating the target have on the cellular phenotype that we would be modulating (proof of principle)? Did induction of this phenotypic change result in therapeutic benefit for patients, and at what doses would this occur (proof of concept)? Could we identify AML patients who might benefit from the agent, either by a gene mutational change or an over/under-expression of the protein target?

In this thesis, we attempted to answer some of these questions. In chapter I, we outlined the current controversies in the treatment of AML and reviewed the literature relating to the Nedd8 conjugation pathway as it applied to oncogenesis and potential for therapeutics. This work led to 2 publications and provided a strong foundation of knowledge to explore the activity of MLN4924 in a well established pre-clinical model of AML [1, 116] (**APPENDIX A & B**).

In chapter 2, we documented for the first time, the potent anti-leukemia activity of MLN4924 in cell lines, primary patient material and xenograft animal models [50] (**APPENDIX C**). We showed this new agent had low nano-molar efficacy across a diverse range of AML phenotypes and that the apoptotic effect of this drug was selective for leukemic blasts. In our studies, AML cells retained their sensitivity to MLN4924 despite signaling from *flt3* mutations and bone marrow stromal cells, both common causes of drug resistance in vivo. Several potential mechanisms explaining the AML cell death observed were considered. Numerous protein substrates accumulated in cells as a consequence of impaired degradation. Could some of these proteins possess anti-proliferative activity? Could impaired protein degradation be leading to ER stress and oxidative damage in a similar way to what is observed with non-selective disruption of protein homeostasis (eg proteasome inhibition)? Might there be specific pathways important for AML survival which were being regulated by nedd8 conjugation? In view of the early induction of pIkB in treated cells, we tested the hypothesis that abnormal NFkB signaling was leading to apoptotic cell death. We showed that through reduced SOD2 levels (an NFkB target gene), that treated cells were being primed for apoptosis through increased generation of reactive oxygen species. These cells could be partially protected from the effects of MLN4924 by pre-treating them with the anti-oxidant enzyme NAC, indicating that there were other mechanisms in addition to altered cellular redox, mediating the drug effect. A more thorough understanding of the multi-factorial effects of this molecule will be the subject of future studies. Our pre-clinical work was published in *Blood* and was the first report of MLN4924 activity in AML models [50]. This work justified the conduct of a phase I clinical trial of a first in class molecule, in patients with advanced MDS and AML.

In chapter 3 of the thesis, we outline an interim analysis of the clinical activity of MLN4924 [97]. Patients were treated with escalating doses 3 times a week every 3 weeks. We defined the MTD for MLN4924 at this schedule and found that liver toxicity was dose limiting. We treated a further 10 patients at the MTD and reported no additional DLTs. We showed that MLN4924 was remarkably well tolerated. In general there was very little stem cell toxicity as we and others had predicted from pre-clinical studies [50,

104]. Of the extra-medullary toxicities we reported which were directly attributable to the drug, these were generally grade 1-2 events and included infusion reactions (fever), muscle cramps and transaminitis. We showed that plasma drug levels of MLN4924 rose rapidly following infusion and declined in a biphasic fashion. The estimated half life of this agent was between 4 and 9 hours. Based on our pre-clinical data we were able to show the consequences of NAE inhibition in treated patients. Lastly, despite very aggressive biology, we reported impressive clinical activity for MLN4924 and documented a CR rate of 16% (n=4) in 24 patients treated. These findings were presented at an oral session at ASH 2010 [97] (**APPENDIX D**). We expect this trial to meet its accrual objectives in the summer of 2012 and hope to publish the final manuscript at the end of the same year.

Finally, research into protein homeostasis and the Nedd8 conjugation pathway has progressed rapidly over the course of the last decade. However, there are still many unresolved issues surrounding the use of neddylation inhibitors in the clinic and the exact role that protein neddylation plays in AML biology. Although this thesis contributes to new knowledge in the exciting arena of AML experimental therapeutics, much work is needed to optimize clinically meaningful biomarkers, uncover mechanisms of resistance to NAE inhibition and design effective and rational combination studies. These efforts, it is hoped, will translate in the development of better therapies that will prolong the lives of patients with AML.

# Appendix A

## Personalized medicine for acute myelogenous leukemia—At the entrance gate

Ronan T. Swords,<sup>1</sup> Bruce J. Dezube,<sup>2</sup> and Bruno C. Medeiros<sup>3\*</sup>

With the advent of molecularly targeted therapies in the oncology clinic, previously fatal blood cancers have now become chronic diseases. Imatinib, a small molecule tyrosine kinase inhibitor that targets the BCR-ABL fusion protein, dramatically extends survival for patients with chronic myelogenous leukemia and has opened the flood gates for modern "personalized cancer medicine". Rituximab, a monoclonal antibody with affinity for the CD20 antigen of B-lymphocytes, in an analogous fashion has revolutionized treatment for patients with B-cell lymphomas. Does this paradigm, a rather commonplace approach for the treatment of hematologic malignancies, apply also to acute myelogenous leukemia (AML)? This is an exciting time to be involved in AML research, and an explosion of new basic science discoveries has formed a springboard for some brand new personalized treatment concepts that this commentary will serve to outline.

### Targeted Therapy

A target worth pursuing should be one that is essential in generating or maintaining the malignant state and at the same time, be amenable to inhibition without causing excessive toxicity to normal cells. An eclectic range of these "druggable" opportunities exist in acute myelogenous leukemia (AML). Protein neddylation involves the conjugation of NEDD8 proteins to target substrates, and this pathway regulates the post-translational modification of several proteins important in the survival of AML cells in laboratory models. Neddylation inhibitors (MLN4924) are now being evaluated in the clinic [1].

The X-linked inhibitor of apoptosis proteins (XIAP) confers chemoresistance in AML cell lines. Blockade of this target with XIAP antisense oligonucleotides reduces XIAP mRNA (AEG35156) and may improve the activity of standard AML chemotherapy [2]. Other resistance mechanisms, which have been more extensively investigated in AML, such as over-expression of drug efflux pumps, are still ripe for further clinical investigation.

The Wilms' tumor 1 protein (WT1), a nearly universal tumor antigen, is an exciting new immunotherapeutic target because of its established role in leukemogenesis and superior immunogenic characteristics. Immunization of patients using WT1 antigen-loaded dendritic cells holds promise for adjuvant treatment of AML after standard therapy [3]. Other targeted approaches include reactivation of the tumor suppressor protein p53, a powerful growth suppressive and proapoptotic molecule frequently inactivated in cancer by gene mutations or defective signaling. Activation of p53 by antagonism of its negative regulator MDM2 has been proposed as a novel strategy for AML therapy and RG7112, a member of the Nutlin family of MDM2 antagonists, appears to be safe and effective in early clinical studies [4].

A vast array of constitutively activated protein kinases lead to inappropriate proliferation and prolong survival of AML cells and many of these are fair game for cancer therapeutics. Probably, the most studied of these has been FLT3, a receptor tyrosine kinase which is mutated in a third of patients with AML and which generally heralds a bad prognosis. The available inhibitors tested in

the clinic have not been as exciting as anticipated however, second generation drugs like AC220 warrant further investigation [5].

The monoclonal antibody (mAb) gemtuzumab ozogamicin, targeting the universally expressed CD33 protein on the surface of AML cells, was recently withdrawn by the FDA due to higher rates of fatal toxicity in randomized studies [6]. Nonetheless, there is still some merit to using other monoclonal antibodies in the clinic. Proteins like CD123 and CD47 might be more viable targets since these are expressed on the surface of so-called leukemia-initiating cells where CD33 is not [7]. In addition, attaching alpha-emitting radioisotopes or protein toxins to rationally designed mAbs may be other ways to increase the therapeutic index of these agents.

Newer insights into AML biology including cell stress mechanisms such as unfolded protein check points, redox balance and metabolic susceptibility (e.g., fatty acid biosynthesis and glucose metabolism) can now also be considered as new targets for drug discovery.

### The Leukemic Stem Cell

The long established view that AML clones are derived from biologically distinct leukemia initiating cells (LICs) has been borne out by several lines of evidence. These unique leukemia stem cells have a distinct phenotype, different from that of normal myeloid stem cells and early progenitor cells. In some cases, more than one LIC subpopulation may be found in the leukemic environment. These cells, like most normal stem cells, will lie dormant in a quiescent pool. As a consequence, the enthusiasm for using cell cycle dependant cytotoxic drugs is diminished. Therefore, agents that eliminate these cells regardless of their cell cycle activity are attractive, or alternately, agents that commit them to cell division may help augment the efficacy of existing cytotoxics. The signaling pathways important for the survival and cellular fate of these LICs have obvious therapeutic implications. The NF- $\kappa$ B, PI3/Akt/mTOR, and Wnt/ $\beta$ -catenin pathways appear to be intrinsically important in LIC survival and many of the agents that disrupt these pathways are already in clinical development for other indications [7]. In addition to the

<sup>1</sup>Institute for Drug Development, Office of the Director, Cancer Therapy and Research Center at the University of Texas Health Science Center at San Antonio, San Antonio, Texas; <sup>2</sup>Millennium Pharmaceuticals, Inc., Cambridge, Massachusetts; <sup>3</sup>Department of Medicine, Division of Hematology, Stanford University School of Medicine, Stanford, California

Conflict of interest: Dr. Swords reported that he has received research funds from Millennium Pharmaceuticals, Inc. and Dr. Medeiros reported that he has received research and consulting funds from Millennium Pharmaceuticals, Inc. Novartis and Celgene Co. Dr. Dezube is an employee of Millennium Pharmaceuticals, Inc. No other disclosures were reported.

\*Correspondence to: Bruno C. Medeiros, M.D. - Stanford Cancer Center, 875 Blake Wilbur Drive, Stanford, CA 94305-5821.  
E-mail: bruno.medeiros@stanford.edu

Received for publication 28 March 2011; Accepted 12 April 2011

Am. J. Hematol. 86:631–632, 2011.

Published online 19 April 2011 in Wiley Online Library (wileyonlinelibrary.com).  
DOI: 10.1002/ajh.22060

## commentary

intrinsic features of LICs, numerous agents are now in development and exert their cytotoxic activity by disrupting the relationship the LIC has with its microenvironment, antiangiogenic drugs being the more established of these. Newer drugs like plerixafor, inhibit the LIC/stromal (CXCR4/SDF-1) interaction and eliminate extrinsic survival signals overcoming drug resistance [8].

### New Insights from Molecular Genetics

Multiple submicroscopic genetic alterations with prognostic significance have recently been discovered in AML and account for some of the heterogeneity within this disease. Genome-wide expression signatures have now allowed us to separate out newly diagnosed patients into separate previously unrecognized biologic and/or prognostic subgroups [9]. These discoveries will certainly have management implications because many of the identified genetic alterations not only represent independent prognosticators and predictors of response to specific therapies, but also represent new targets for treatment. Next generation sequencing technologies will analyze diagnostic material in real time and based on individual changes in a patients' genome/transcriptome should allow clinicians to decide on appropriate selection of the treatments outlined above. In addition, existing therapies in AML, which have been disappointing in the clinic (e.g., epigenetic agents) may become more relevant as our ability to identify sensitive populations improves.

### Conclusions

The challenge in AML therapeutics is clear. Conventional treatments for this disease are the same in many of their particulars now as they were 3 decades ago. Only about 40% of patients considered to have an "intermediate" prognosis will be cured with these drugs. The tools of modern cancer cell biology have presented us with an exciting array of new treatment concepts, all of which merit clinical investigation. Advances in clinical trial design to confine Phase III studies to the target population will lead to the

success of these new therapies. Personalized cancer medicine for AML is here and offers real hope for the cure of this disease.

### Acknowledgements

Authors would like to thank Francis J. Giles, MD who provided valuable feedback as the content of this Commentary was developed and for critical review of its final version. No compensation was received.

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## Expert Opinion

1. Introduction
2. The NEDD8 conjugation pathway
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4. NAE as a new target for new drug development
5. Conclusions
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## Targeting protein neddylation: a novel therapeutic strategy for the treatment of cancer

Meng Wang, Bruno C Madeiros, Harry P Erba, Daniel J DeAngelo, Francis J Giles & Ronan T Swords<sup>†</sup>

<sup>†</sup>*Cancer Therapy and Research Center, Hematology/Oncology, 7979 Wurzbach Road, SA, Texas 78230, USA*

**Introduction:** The NEDD8 (neural precursor cell-expressed developmentally downregulated 8) conjugation pathway regulates the post-translational modification of oncogenic proteins. This pathway has important potential for cancer therapeutics. Several proteins vital in cancer biology are regulated by protein neddylation. These observations led to the development of a small molecule inhibitor that disrupts protein neddylation and leads to cancer cell death and important activity in early phase clinical trials.

**Areas covered:** This review provides an extensive coverage of cellular protein homeostasis with particular emphasis on the NEDD8 conjugation pathway. Insights into a new investigational drug that specifically disrupts the NEDD8 pathway are discussed. The clinical data for this agent are also updated.

**Expert opinion:** Neddylation controls key cellular pathways found to be dysregulated in many cancers. Protein neddylation is a relatively under-explored pathway for pharmacologic inhibition in cancer. Selective disruption of this pathway has demonstrated clinical activity in patients with myeloid neoplasms and is worth exploring further in combination with other anti-leukemia agents.

**Keywords:** acute myeloid leukemia, MLN4924, neddylation, therapy

*Expert Opin. Ther. Targets (2011) 15(3):1-11*

### 1. Introduction

An improved understanding of how changes in protein homeostasis can drive the pathogenesis of many human diseases has provided the platform for the discovery of several important anticancer therapies. The discovery of the ABL kinase as the onco-protein driving proliferation in chronic myeloid leukemia [1,2] led to the development of imatinib mesylate (Gleevec<sup>®</sup>, Novartis, East Hanover, NJ, USA), the small molecule inhibitor that revolutionized experimental therapeutics in cancer [3,4]. Now, in continuing the era of targeted therapy, a clearer understanding of the NEDD8 conjugation pathway has presented novel targets for the development of new anticancer agents. Protein neddylation is catalyzed by E1, E2 and E3 enzymes which conjugate the ubiquitin-like protein NEDD8 (neural precursor cell-expressed developmentally downregulated 8) to a diverse range of target substrates [5-8]. The NEDD8 gene was first described from work conducted on the developing mouse brain where its activity was shown to be downregulated [9]. Subsequent identification of intracellular substrates conjugated with NEDD8 has given us significant insight into the cellular pathways regulated by this protein. The best-characterized function of NEDD8 is the activation of the cullin-RING family of E3 ubiquitin-ligases (CRLs), which polyubiquitinates proteins and targets them for degradation by the proteasome. Because many CRL substrates

#### Article highlights.

- The NEDD8 (neural precursor cell-expressed developmentally downregulated 8) conjugation pathway, similar in many ways to the ubiquitin proteasome system, involves the sequential transfer of a ubiquitin-like protein (NEDD8) across E1, E2 and E3 enzymes before conjugation to a target substrate.
- The single-most characterized function of NEDD8 is in the activation of the cullin RING ligases, which are E3 ubiquitin ligases that polyubiquitinate protein substrates with important functions in cell cycle regulation, signal transduction, DNA damage and the cellular stress response.
- Protein neddylation has been shown to play critical roles in the maintenance of cancer cell survival and, therefore, represents an important new pathway for cancer therapeutics.
- Genetic and pharmacologic disruption of the NEDD8 conjugation pathway result in the accumulation of cullin RING ligase-dependent substrates and cause cancer cell death through several different mechanisms, which include alteration of cellular redox status and disruption of the cell cycle.
- The novel small molecule inhibitor MLN4924 (Millennium Pharmaceuticals) forms a covalent complex with NEDD8; this complex prevents the ATP-dependent activation of NEDD8 by its E1 activating enzyme NEDD8 activating enzyme and blocks NEDD8 conjugation with target substrates in a unique way.
- MLN4924 has shown potent preclinical activity in a number of histologies including prostate cancer, breast cancer, myeloma, lymphoma and acute myeloid leukemia (AML).
- MLN4924 is the first specific inhibitor of the NEDD8 conjugation pathway in clinical development. Its activity appears to be most impressive in patients with AML.
- Protein neddylation is a relatively under-explored pathway for pharmacologic inhibition in cancer. Selective disruption of this pathway has demonstrated clinical activity in patients with myeloid neoplasms and is worth exploring further in combination with other anti-leukemia agents.

This box summarizes key points contained in the article.

have tumor suppressor activity with important roles in cell-cycle progression, DNA damage and the cellular stress response, preventing the degradation of these proteins could prove to be an effective anticancer strategy [10-14]. Of course, targeting protein degradation has already been validated as an effective anticancer therapy and led to the approval of the proteasome inhibitor bortezomib (Velcade®, Millennium, Cambridge, MA, USA) for clinical use in myeloma and lymphoma [15,16]. However, this agent lacks specificity in that it prevents the degradation of all polyubiquitin-conjugated proteins which may contribute to clinical toxicity. More selective inhibition of protein degradation may allow for the design of drugs that have similar anticancer activity but with improved

tolerability. MLN4924, a first in class small molecule inhibitor developed by Millennium Pharmaceuticals, was recently reported as a specific inhibitor of protein neddylation [12-14,17].

This review summarizes current understanding of protein neddylation in the regulation of cellular function and the translational medicine that has emerged as a result of a broader understanding of this process.

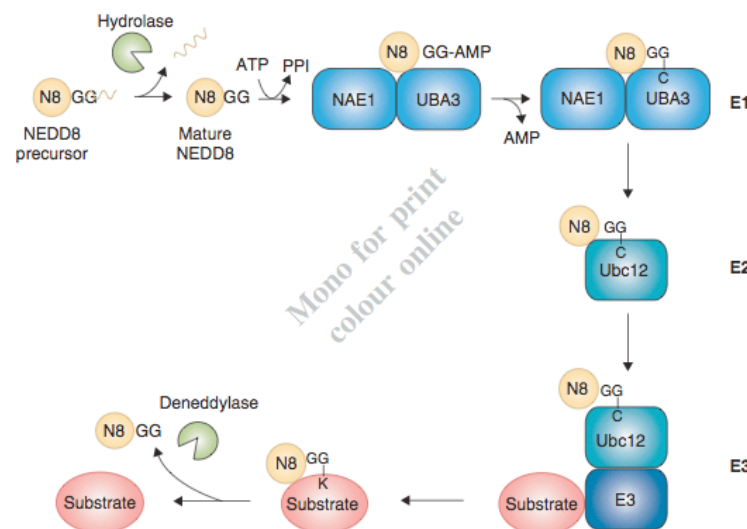
## 2. The NEDD8 conjugation pathway

### 2.1 Processing and conjugation of NEDD8

Conjugation of NEDD8 to target proteins involves the coordinated and sequential transfer of NEDD8 between a series of enzymes (E1, E2 and E3 enzymes) before being attached to a target substrate (Figure 1). NEDD8 is synthesized as an 81-amino-acid precursor, which cannot be used in conjugation reactions until hydrolases (UCH-L3 or DEN1) cleave away a portion of the C terminus to reveal a glycine residue (Gly 76) to which NEDD8 is covalently attached. The processed NEDD8 is initially activated by the NEDD8 activating enzyme (NAE). This enzyme serves as the E1 activating enzyme and consists of a heterodimer comprising NAE1 and UBA3 (ubiquitin-like modifier activating enzyme 3) subunits. NAE activation of NEDD8 is an ATP-dependent reaction which forms a NEDD8-AMP intermediate before NEDD8 is transferred to a cysteine residue in the NAE active site via a thioester bond. The NAE-NEDD8 complex then transfers NEDD8 to an E2 conjugating enzyme. Two of these enzymes have been identified, Ubc12 (also known as UBE2M) and UBE2F [18]. The final step in the process involves the interaction of an E2 with an E3 ligase to conjugate NEDD8 to lysine residues on the substrate protein. The E1 and E2 enzymes that catalyze the neddylation reaction are specific to NEDD8, thus, preventing nonspecific conjugation with ubiquitin or other ubiquitin-like proteins [5,19-26]. Several NEDD8 E3 ligases have been proposed to date (Table 1). Deneddylation by NEDD8 isopeptidases such as CSN5 (a component of the COP9 signalosome and responsible for cullin deneddylation) and DEN11 (also known as NEDP1 or SENP8) helps regulate the loading of NEDD8 on and off protein targets [5].

### 2.2 Cellular functions of neddylation

Deficiency of components in the neddylation pathway resulted in a lethal phenotype in several genetic models, thus, revealing little detail regarding the role of neddylation in cellular function other than that NEDD8 is essential for embryogenesis and cell viability [19,27-31]. The identification of protein substrates that can be modified with NEDD8 eventually enabled a more detailed study of how neddylation affects cellular pathways. In a seminal review of the Nedd8 pathway, Rabut and Peter [5] provide several criteria to determine whether a protein is a Nedd8 target. Based on these criteria, protein substrates should be: i) covalently attached to Nedd8, ii) detectable under endogenous



**Figure 1. The neddylation pathway.** The processing and the conjugation of NEDD8 onto its substrate. NEDD8 is initially synthesized as a precursor with a C-terminal tail that is cleaved away by NEDD8 hydrolase. This produces the mature form of NEDD8 which can be conjugated onto substrates via the glycine residues exposed at the C terminus of the NEDD8 molecule. The E1 enzyme, also known as the NEDD8 activating enzyme, a heterodimer formed by NAE1 and UBA3 subunits, binds the mature NEDD8 and an ATP to catalyze the formation of a NEDD8-AMP intermediate. This NEDD8-AMP intermediate then reacts with the thiol group of a cysteine molecule in the active site to form an E1-NEDD8 thioester. This form of the E1 enzyme interacts and transfers the NEDD8 to an E2 enzyme (Ubc12 is depicted in this figure, Ube2F may also be utilized). A specific E3 enzyme recruits the E2-NEDD8 complex and mediates the conjugation of NEDD8 onto a lysine residue of the substrate. In the case of NEDD8 charged Ubc12, NEDD8 may be directly transferred to cullin substrates. The NEDD8 can be removed from the substrate by specific deneddylase enzymes.

NAE1: NEDD8 activating enzyme 1; NEDD8: Neural precursor cell-expressed developmentally downregulated 8; UBA3: Ubiquitin-like modifier activating enzyme 3.

**Table 1. Proposed neddylated substrates and their functions.**

Neddylated protein	NEDD8 ligase	Effect of neddylation
CUL1, 2, 3, 4A, 4B, 5, 7, Parc [32,57-61]	Rbx1/Dcn1	Enhances E3 ubiquitin ligase activity
p53 [7,62]	Mdm2/FBX011	Inhibition of p53 transcriptional activity by localization into the cytoplasm
p73 [38]	Mdm2	Inhibition of p73 transcriptional activity by localization into the cytoplasm
Mdm2 [7]	Mdm2	Reduces the inhibitory activity of Mdm2 on p53, whilst another report suggests neddylated Mdm2 exhibits enhanced stability
EGFR [63]	c-Cbl	Enhancement of EGFR ubiquitination and its subsequent lysosomal degradation
AICD [64]	Unknown	Inhibition of AICD transcriptional activity by preventing its interaction with transcriptional co-activators Fe65 and Tip60
pVHL[23,65]	Unknown	Promotes interaction of pVHL with fibronectin
BCA3 [66]	Unknown	Recruitment of SIRT1 HDAC to inhibit NF-κB transcriptional activity
L11 and other ribosomal proteins [8]	Mdm2	Enhances stability and promotes localization to nucleolus

conditions, iii) dependant on NAE1 and Ubc12 or UBE2F, iv) have a neddylated lysine residue, v) not be available for attachment to Nedd8 when mutated, vi) be associated with a defined E3 ligase and vii) be associated with deneddylase activity. The currently known neddylated substrates are

summarized in Table 1; however, it should be noted that based on the listed criteria, the only bona fide substrates for Nedd8 are the E3 cullin RING ligases (Cul1, Cul2, Cul3, Cul4A, Cul4B and Cul5). Below, we discuss the cullin E3 ligases (as the best characterized neddylated



substrates) and p53 (given the relevance of this protein in cancer biology).

### 2.2.1 Cullin-RING E3 ubiquitin-ligases

The CRLs are a family of E3 ubiquitin ligases consisting of a cullin protein acting as a central scaffold to recruit other subunits to form a modular E3 core complex. An adaptor subunit binds at the N terminus of the cullin to mediate contact with the substrate recognition protein. The Rbx1 protein containing a RING domain binds to the C terminus of the cullin to act as a docking site for ubiquitin-charged E2 ligases. However, efficient ubiquitin ligase activity by CRLs also requires a NEDD8 molecule to be conjugated to the C terminus of the cullin. The addition of NEDD8 in this way is catalyzed through the combined action of Rbx1 and Dcn1 proteins. Neddylation has been proposed to activate ubiquitin ligase activity through several putative mechanisms. NEDD8 arguably could provide a docking site for ubiquitin-activated E2 enzymes (although the evidence for this based on recent structural biology work is weak), induce conformational changes in cullin proteins to bring an E2 closer to its substrate (similarly, the basis for this idea is not clearly substantiated in the literature), promote cullin dimerization and prevent interaction with the E3 ligase inhibitor CAND1 [5,20,21]. Several cullin proteins have been described in humans (CUL1, CUL2, CUL3, CUL4A, CUL4B, CUL5 and CUL7) in addition to two cullin-like proteins (PARC and APC). Most of the cullin proteins appear to be neddylated which influence cullin function and substrate selection for degradation [32]. For example, CUL1 catalyzes the ubiquitination of p27, phosphorylated I $\kappa$ B- $\alpha$ ,  $\beta$ -catenin, c-myc, c-jun and several others [10]. CUL2 and CUL5 target HIF1- $\alpha$ , CUL3 targets NRF2 and CUL4A targets CDT1 and p27 [32]. Many of these cullin substrates of course play critical roles in oncogenesis.

### 2.2.2 The p53 pathway

In response to cellular stress, oncogene activation or DNA damage, the p53 tumor suppressor activates the transcription of target genes that result in cell cycle arrest, apoptosis or senescence [33,34]. These important functions of p53 in maintaining genome fidelity are clearly demonstrated by finding mutations of this protein in > 50% of human cancers [35]. The stability and activity of p53 is dependent on its post-translational modification by Mdm2, an E3 ubiquitin ligase capable of NEDD8 and ubiquitin conjugation to its target. Polyubiquitination of p53 results in its degradation by the proteasome, and it appears that neddylation also negatively regulates p53 by inhibiting its transcriptional activity, possibly through an interaction between NUB1 and NEDD8 that causes neddylated p53 to become localized to the cytoplasm [7,36]. Mdm2 auto-neddylation has been reported and may play a role in the kinetics of p53 degradation [7,37]. Other members of the p53 family are also reported as substrates for neddylation, such as p73, and are regulated in a similar

way [38]. The physiologic significance of p53 neddylation remains controversial, however, and further work is needed to fully understand the role of Nedd8 in p53 function.

## 3. Rationale for a NEDD8 inhibitor

Several factors are important for consideration when selecting molecular pathways as targets for anticancer drug development. The pathway must be critical for the survival of the cancer cell, subject to mutation or deregulation, disruption of the pathway should not be toxic to normal cells and the pathway must be amenable to pharmacologic manipulation. Given these requirements for rational drug design, the NEDD8 conjugation pathway becomes an exciting pathway for the development of novel agents. Although protein neddylation appears to be critical for some genetic systems [27-31] (which is of concern when exploiting the pathway for therapeutics in humans), pharmacologic deregulation of the NEDD8 conjugation pathway appears to have a selective anticancer effect and is well tolerated in early clinical studies, as is outlined later.

Numerous reports support the importance of the NEDD8 pathway in cancer. Levels of neddylated protein substrates are higher in cancer cell lines when compared to normal fibroblasts and aberrant NEDD8 conjugation in cancer cells has been shown [39]. In addition, transfection of the oral carcinoma cell line HSC4 with a dominant negative Ubc12 which inhibited endogenous neddylation was shown to have anti-proliferative effects, suggesting that aberrantly increased neddylation contributes to the increased cell proliferation seen in cancer cells. Others have shown the association of increased levels of neddylated cullins with high grade neuroendocrine lung carcinomas [40].

Several NEDD8-dependent pathways have been clearly shown to be critical for the growth and survival of a variety of cancer models, many of these pathways are already the focus of drug development. Among these, targeting the NF- $\kappa$ B pathway has had the greatest success in the clinic. Drugs such as bortezomib and its analogs indicate that there is considerable therapeutic potential in the development of agents capable of more selective inhibition of the ubiquitin-proteasome system. Disruption of the p53-NEDD8 pathway could potentially enhance p53 function, resulting in anticancer activity through augmented cell cycle arrest and apoptosis. It is well recognized in p53-deficient mice that restoration of activity leads to increased senescence and apoptosis [41-43]. However, as we have outlined previously, the link between Nedd8 and p53 is not fully characterized and targeting the Nedd8/p53 pathway clearly requires the presence of wild-type p53, which of course is mutated or nonfunctional in many patients with cancer which might limit this approach [34].

Crystallography studies of the NAE in complex with its substrates have led to important insights into relevant protein-protein interactions and the catalytic mechanism of

the E1 enzyme carrying out the initial neddylation reaction [11,17]. This has allowed for the design of small molecules that disrupt the interaction of NAE with its binding partners. A neddylation inhibitor could potentially have a better toxicity/efficacy profile as compared to a proteasome inhibitor because proteasome inhibition affects the metabolism of all polyubiquitinated proteins as opposed to a neddylation inhibitor that would only affect proteins that are polyubiquitinated by CRLs. Because several anti-proliferative proteins (e.g., p27, I $\kappa$ B $\alpha$ ) are tagged with polyubiquitin by CRLs which leads to their destruction, a NAE1 inhibitor should increase the levels of p27, I $\kappa$ B $\alpha$  and others thereby blocking cell proliferation. A proteasome inhibitor would not only affect these proteins, but all other regulatory proteins (proteins that are either pro- or anti-proliferative and/or have nothing to do with cell proliferation) as well as house-keeping proteins (protein that need to be turned over due to normal wear and tear) that are destroyed by the proteasome. One could make an argument that a neddylation inhibitor, by virtue of the fact that it affects the metabolism of critical anti-proliferative proteins, would exact a more selective antitumor effect than a proteasome inhibitor.

#### 4. NAE as a new target for new drug development

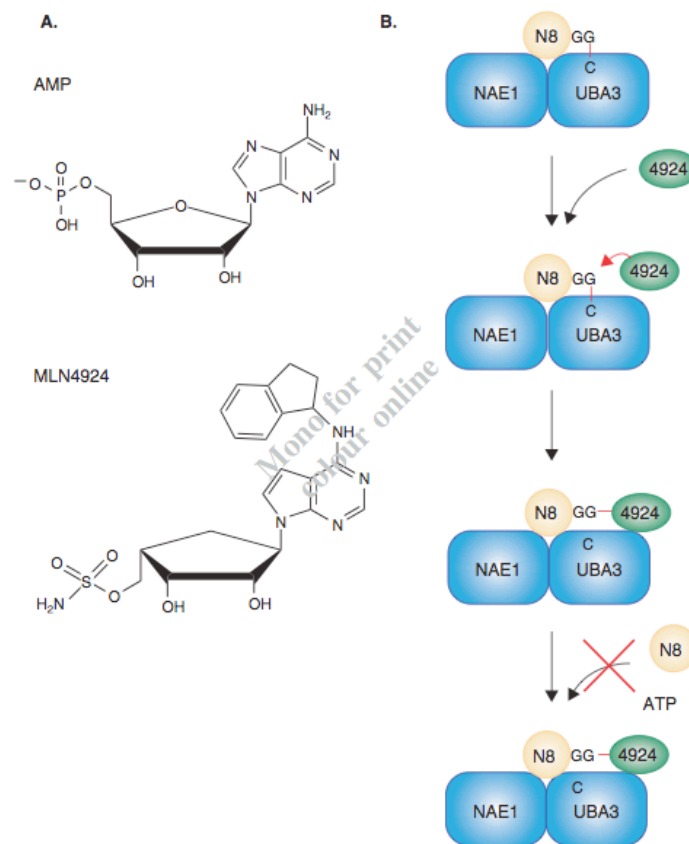
Ubiquitin and NEDD8 conjugation share similar characteristics. The E1-activating enzymes for both pathways [24] require ATP binding to form an acyl-adenylate intermediate. The resulting Ub-AMP or NEDD8-AMP intermediate then reacts with a cysteine residue in the active site to form an E1-Ub or an E1-NEDD8 complex, before interaction with an E2 takes place. E1 ubiquitin-activating enzymes (UAEs) can be inhibited by hydrolytically stable AMP analogs such as adenosylphospho-ubiquitinol, which bind to active sites and prevent the formation of Ub-AMPs [44]. Due to the size of these analogs, clinical development was not possible because they could not be delivered into cells. Nevertheless, the conservation of the reaction mechanism between ubiquitin and NEDD8 E1 enzymes suggested that a similar strategy could be utilized to design an inhibitor of NAE.

High-throughput screening technology discovered N6-benzyl adenosine as an inhibitor of NAE that with additional iterative medicinal chemistry efforts resulted in the identification of MLN4924 (Figure 2A) [12]. MLN4924 (Millennium Pharmaceuticals) is a potent and highly selective inhibitor of its target *in vitro* (IC<sub>50</sub> 4 nM) when compared to other E1 activating enzymes (IC<sub>50</sub>: UAE, 1.5  $\mu$ M; SMO activating enzyme, 8.2  $\mu$ M; UBA6, 1.8  $\mu$ M and autophagy related protein 7, > 10  $\mu$ M) or ATP-dependent protein kinases [12]. Crystallography data have revealed that MLN4924 inhibits NAE via the formation of a NEDD8-MLN4924 adduct. In the first step of the NAE reaction, ATP and NEDD8 bind with the release of inorganic pyrophosphate. A NEDD8-AMP intermediate is then formed which reacts

with cysteine residues in the enzymes active site with the release of AMP. In the last step, a second round of substrates bind and produce a ternary complex where the enzyme has two bound NEDD8 proteins, one as a NEDD8-AMP intermediate and the other as a covalently bound NEDD8 thioester. This species is capable of transferring NEDD8 to an E2 conjugating enzyme via a *trans*-thiolation reaction. MLN4924 functions by occupying the nucleotide binding pocket of the NAE-NEDD8 thioester and reacts with thioester bound NEDD8 to form an MLN4924-NEDD8 adduct. In this form, no further ATP binding can occur and NAE activation of NEDD8 is disrupted, thereby, preventing conjugation with target proteins (Figure 2B) [11]. Unlike the larger AMP analogs necessary for UAE inhibition, the MLN4924 AMP analog is small enough to reach its intracellular target (Figure 3).

#### 4.1 Preclinical studies

The activity of MLN4924 has been explored across several hematologic and solid tumor *in vitro* models with variable sensitivity (IC<sub>50</sub> – 0.01 to 1  $\mu$ M) (Table 2). In preclinical acute myeloid leukemia (AML) models, MLN4924 selectively overcomes the effect of stromal-mediated signaling, previously proposed as a mechanism of chemoresistance to other anticancer agents [14,45]. Administration of MLN4924 to mice bearing xenografts of both hematologic and solid tumors led to stable disease regression at doses and schedules which were well tolerated [12-14]. Treatment with MLN4924 results in a rapid change in neddylation status *in vitro* and *in vivo*. Significantly decreased levels of neddylated cullins were observed as early as 30 min after exposure to MLN4924 [11-14,17] with the subsequent accumulation of cullin-dependent substrates (p27, CDT-1, NRF-2 and phosphorylated I $\kappa$ B $\alpha$ ) but not non-CRL substrates [12-14,17]. Minimal inhibition of protein turnover in cells treated with MLN4924 was observed when compared to the same cells treated with bortezomib, consistent with the observation that this agent offers more selective disruption of protein degradation [12,13]. Several mechanisms of action for this compound have been proposed. In AML and the activated B-cell-like subtype of diffuse large B-cell lymphoma, exposure to MLN4924 triggered apoptosis primarily following the downregulation of the NF- $\kappa$ B pathway. Increased levels of phosphorylated I $\kappa$ B $\alpha$  decreased DNA binding activity of NF- $\kappa$ B and reduced levels of NF- $\kappa$ B target genes, for example, BCL-2, BCL-xL, FLIP and SOD2 [14,17]. In AML models, reduced levels of the antioxidant enzyme SOD2 was associated with increased generation of reactive oxygen species in a dose-dependent fashion [14]. In solid tumor and lymphoma cell lines, S-phase arrest with subsequent accumulation of DNA caused cells to die by apoptosis [13,17]. This defect correlated with the accumulation of the DNA replication licensing factor CDT-1, which has been previously shown to cause multiple rounds of DNA replication when overexpressed [12]. The exact mechanisms underlying the cytotoxicity of MLN4924 are likely to be multifactorial



**Figure 2. Inhibition of NEDD8 activating enzyme by MLN4924. A.** Structure of AMP in comparison to the AMP analog MLN4924. **B.** MLN4924 binds to the AMP site in the E1 enzyme and the sulfamate group in MLN4924 attacks the thioester bond between NEDD8 and the cysteine of the E1 making an MLN4924-NEDD8 adduct which is stable and inhibits further reactions.

NEDD8: Neural precursor cell-expressed developmentally downregulated 8.

and may also depend on the status of other signaling pathways within the cell, which will require further experimentation to unravel.

#### 4.2 Clinical studies

MLN4924 is being evaluated in early phase clinical studies in advanced solid tumors (C15001), lymphoma and myeloma (C15002), melanoma (C15005), and in AML and advanced myelodysplastic syndromes (C15003). Preliminary data presented at ASCO in 2009 from the C15001 protocol indicated that treatment with MLN4924 resulted in accumulation of substrates associated with target inhibition in both peripheral blood and skin. Also, plasma drug levels were reached that exceeded concentrations required for *in vivo* antitumor activity in xenograft models [46]. At ASH 2009,

Shah *et al.* [47] presented an interim analysis of 22 patients enrolled on the C15002 protocol (14 with myeloma, 8 with non-Hodgkin's lymphoma). Six dose levels were evaluated and four patients experienced a dose-limiting toxicity: one grade 4 febrile neutropenia, one grade 3 liver impaired liver function, one grade 4 muscle cramp and one grade 2 myalgia that was considered dose limiting. The most common adverse events (AEs) included fatigue, nausea, myalgia and elevated liver enzymes. Myelosuppression was infrequent. No toxic deaths were reported in the C15002 trial. MLN4924 displayed a multiexponential pharmacokinetic (PK) profile with a half-life of 4 – 9 h, relatively low PK variability and approximately dose-proportional increases in total plasma exposure. Pharmacodynamic evaluation confirmed target inhibition. These data were updated at the European





**Figure 3. MLN4924 target inhibition.** Structural rendering of MLN4924 occupying nucleotide binding pocket of NAEβ. Image courtesy of Mike Sintchak, Millennium Pharmaceuticals.

NAE: NEDD8 activating enzyme; NEDD8: Neural precursor cell-expressed developmentally downregulated 8.

Hematology Association earlier this year [48]. On the C15003 protocol, 15 patients have been enrolled to date. Two dose-limiting toxicities have been reported at 78 mg/m<sup>2</sup>. In this study, MLN4924 was given as an intravenous infusion on days 1, 3 and 5 of a 21 day cycle. Two dose-limiting toxicities have been reported at 78 mg/m<sup>2</sup>. One patient had multi-organ failure in the context of sepsis (after 2 cycles), the other had reversible elevation of alanine aminotransferase during cycle 1 which resolved after withdrawal from study. The most common AEs were pneumonia (n = 6), atelectasis, constipation, diarrhea and febrile neutropenia (each n = 4); most common grade ≥ 3 AEs were febrile neutropenia (n = 4), elevated aspartate aminotransferase and pneumonia (each n = 3). Three patients achieved a complete response (CR). A 29-year-old woman with relapsed AML following allogeneic stem cell transplantation achieved a CR after cycle 1 at 25 mg/m<sup>2</sup> before developing progressive disease at an extramedullary site during cycle 8. An 82-year-old male patient with high-risk MDS/AML who was unresponsive to azacitidine had a partial response in cycle 8 and a CR with incomplete recovery of blood counts (CRi) in cycle 10 at 33 mg/m<sup>2</sup>; the patient was rendered transfusion-independent after 12 cycles of treatment. A 71-year-old man with *de novo* AML refractory to standard cytarabine plus daunorubicin induction achieved a CRi during cycle 1 at 44 mg/m<sup>2</sup>; although this was not maintained, the patient continued to benefit from treatment with reduced transfusion dependence. Pharmacodynamic data for nine patients revealed that seven showed evidence of target inhibition in peripheral blood by changes in NAE-regulated proteins (unpublished data).

## 5. Conclusions

Research exploring the ubiquitin proteasome system (UPS) has led to a thorough description of neddylation, NEDD8 targets and the structure-function relationship of NEDD8 with the CRLs. The description of this pathway has led to the evaluation of new anticancer drugs targeting both directly and indirectly the E3 ligases that signal upstream of the proteasome. The identification of new cancer pathways is always exciting news; however, it is prudent to remember that many promising drug candidates have been shown to cure cancer in mice, only to fail spectacularly in humans. Ultimately, the success of selective inhibition of the UPS will be revealed from the clinical studies currently accruing and will hopefully lead to another rare event we so desperately seek, the approval of a new anticancer drug!

## 6. Expert opinion

Finding a successful 'hit' in the competitive world of drug development is a rare event. In 2008, the US pharmaceutical industry spent \$65 billion dollars in the pursuit of new drug therapies. In the same year, just one anticancer compound was approved by the FDA [49]. Drug discovery is challenging and the seemingly slow progress of finding new cancer treatments is clearly not due to a lack of investment. Rare also is the identification of a new pathway and a 'druggable target'. These rare events when they occur generate much excitement amongst translational researchers.

The discovery of NAE and the NEDD8 conjugation pathway as one such rare event is rooted in the first description of a protein degrading complex we now refer to as the proteasome. Since its debut in the 1980s it is clear that the UPS is a master regulator of protein homeostasis in cells. Given the biological importance of this system to cancer cells, it was inevitable that the pathway would be exploited for new drug discovery. In 2003, bortezomib was approved as a first in class UPS inhibitor, a successful anticancer drug and a triumph in the fast-track approval of a new molecule from chemical synthesis to use in the clinic [15]. Whilst bortezomib is selective for proteasome inhibition, its effects on proteasome substrate levels are broad ranging, it stabilizes all polyubiquitin-tagged proteins that are destined for elimination and, in general, this type of non-selective activity may be a disadvantage for new agents because many of these substrates are probably not important for efficacy and their abundance may lead to unwanted toxicity. Nonetheless, the huge clinical and commercial success of bortezomib fanned interest in the potential for the development of drugs that target substrate-specific proteins upstream of the proteasome, such as the E3 ligases. Indeed, Roche pharmaceuticals are pursuing the clinical development of *cis*-imidazole derivatives (nicknamed 'Nutlins') that specifically interfere with protein-protein interactions between the E3 ligase MDM2 and its tumor suppressor substrate p53, which is regulated by ubiquitin conjugation. Nutlins have

**Table 2. Summary of effect of MLN4924 on cell lines and murine xenografts.**

Origin	Cell line model	50% Growth inhibition by MLN4924 ( $\mu$ M)	Inhibition of tumor xenograft in mice
DLBCL [17]	WSU-DLCL2	0.06, 0.18	Primary human DLBCL
	HBL-1	0.11	
	L1236	0.2	
	U2932	0.03	
	Farage	0.04, 0.05	
	TOLEDO	0.04, 0.07	
	OCI-Ly3	0.15	
	OCI-Ly10	0.01	
	OCI-Ly7	0.16	
	OCI-Ly19	0.03	
Mediastinal BCL [17]	Karpas 1106P	0.24	OCI-Ly10
Burkitt's lymphoma [17]	Daudi	0.03	
BCL [17]	PA682	0.02, 0.06	OCI-Ly19
	Ramos	0.03, 0.04	
Immunoblastic large cell lymphoma [17]	Pfeiffer	0.01, 0.02	
	RL	0.01, 0.02	
Acute myeloid leukemia [14]	SR	0.02, 0.04	
Myeloma [67]	MOLM-13	0.21	HL60
	PL-21	0.21	
	MV4-11	0.21	
	HL-60	0.21	
	Primary AML Cells	0.3 – 1	
	NCI-H929	0.27	
Colorectal cancer [12,17]	RPMI-8226	0.63	HCT-116
	HCT-116	0.1	
Breast carcinoma [68]	MDA-MB-231	0.48	Calu-6
Lung cancer [12]	Calu-6	0.13	
	H460	1.03	
Prostate cancer [68]	LNCaP	0.05	H522
PBMCs [14]		No inhibition at 3	

BCL: B-cell lymphoma; DLBCL: Diffuse large B-cell lymphoma.

been shown to reactivate the p53 pathway in cancer cell lines resulting in apoptotic cell death [50]. Moreover, they have been shown to suppress the growth of human tumor xenografts in mice without significant toxicity to normal tissues [50,51]. The success of the nutlins in the clinic is uncertain, however, because p53 is non-functional in many cancers and the details of how most E3 ligases actually work are poorly defined, therefore, leading to great uncertainty about how to target them specifically. However, two recent publications have demonstrated the ability to identify specific inhibitors of the SCF E3 ligases (part of the CRL family) giving credence to the potential of selective E3 ligase inhibition as a therapeutic strategy [52,53].

An alternative strategy is to target enzymes upstream of the E3 ligases. One such enzyme is NAE which activates the cullin-RING E3 ligases by conjugation with NEDD8. Over 350 CRLs have been identified in humans controlling the turnover of key substrates that regulate NF- $\kappa$ B signaling, the cell cycle, DNA replication and repair, oxidative stress response pathways and hypoxia signaling [49]. Given these observations, Millennium Pharmaceuticals identified a small

molecule capable of inhibiting NAE in a high-throughput screen that served as a good starting point for a medicinal-chemistry program that led to the birth of MLN4924, a highly potent and selective inhibitor of NAE. The ability of MLN4924 to selectively inhibit protein degradation and cause cancer cell death *in vitro* and *in vivo* highlights the exciting potential of this molecule. Given the involvement of CRLs and consequently neddylation in a myriad of processes required in all cells, it is unclear why cancer cells are more sensitive to NAE inhibition than 'normal' cells. Several mechanisms have been proposed to explain this but consistently it appears that the most frequent cellular consequence of NAE inhibition in cultured cells is the disruption of S-phase regulation leading to DNA damage and cell death.

What therapeutic avenues for targeted manipulation of the UPS could be proposed? One strategy worth exploring would be to combine an NAE inhibitor and another S-phase active agent that might result in a significant increase in DNA damage. NAE depletion is highly toxic to AML cells and it would certainly be of interest to explore the activity of a drug such as MLN4924 in combination with cytarabine, already

a standard of care agent for this histology. It will also be of interest to define the role of the DNA-damage response in determining the sensitivity of cancer cells to NAE inhibition, because many cancer cells have aberrant DNA-damage response pathways. A recent study reported that treatment of cancer cell lines with doxorubicin and camptothecin resulted in the upregulation of the deneddylase enzyme NEDP1, which restored wild-type p53 activity (Watson *et al.*, 2010). In the same study, the loss of NEDP1 reduced susceptibility to DNA damaging agents due to p53 suppression, highlighting the role of NEDP1 in mediating drug resistance [37]. In cancers with NEDP1 upregulation, an NAE inhibitor might combine well with an anthracycline to overcome this effect and induce p53-dependent cancer cell death. Synthetic-lethal RNAi screens that target other cancer pathways in combination with NAE inhibitors will allow further rationalization of combination therapies that may facilitate clinical trial design.

Oncogene addicted cancers that are dependent for their survival by having specific aberrant signaling pathways need to be considered as potentially sensitive to UPS manipulation. For example, ~ 50% of all large cell lymphomas have constitutively active NF- $\kappa$ B signaling [17]. As a consequence of NAE inhibition, the CRL substrate pI $\kappa$ B is stabilized which results in impaired NF- $\kappa$ B signaling and cell death in the large cell lymphoma subtypes that are addicted to this pathway.

Another pathway of interest is the WNT/ $\beta$ -catenin signaling pathway that is activated in colon cancer, some types of breast cancer and hepatocellular cancers. Recent data have linked this pathway to the downregulation of the tumor suppressor p27 via CRL4<sup>DDB1</sup> ubiquitination and degradation [54]. Additionally, WNT/ $\beta$ -catenin signaling is required for self renewal of leukemia stem cells but not for normal hematopoietic stem cells. Quiescent leukemia stem cells are thought to drive leukemia relapses following remission induction therapy [55]. These data suggest that cancers that originate from Wnt-stimulated progenitor cells may be particularly susceptible to NAE inhibition through inhibition of CRL4 activity. It should be noted, however, that the excessive accumulation of the onco-protein  $\beta$ -catenin due to Cull1 inhibition may have adverse effects. Finally, a recent report has implicated the tumor suppressor NF2 as an inhibitor of CRL4 which gives the potential for use of MLN4924 in tumors with mutations in NF2 that result in a dependence on CRL4 activity for survival, for example, malignant mesothelioma [56].

### Declaration of interest

HP Erba and FJ Giles have received research funding from Millennium Pharmaceuticals.

All other authors declare no conflict of interest and have received no payment in preparation of this manuscript.

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#### Affiliation

Meng Wang<sup>1</sup>, Bruno C. Madeiros<sup>2</sup>, Harry P. Erba<sup>3</sup>, Daniel J. DeAngelo<sup>4</sup>, Francis J. Giles<sup>5</sup> & Ronan T. Swords<sup>†6</sup>  
<sup>1</sup>Author for correspondence  
<sup>1</sup>Cambridge University, Medicine, Cambridge, UK  
<sup>2</sup>Stanford, Hematology/Oncology, Palo Alto, CA, USA  
<sup>3</sup>University of Michigan, Hematology/Oncology, Michigan, USA  
<sup>4</sup>Dana Farber Cancer Institute, Boston, USA  
<sup>5</sup>Professor, National University of Ireland Galway, Cancer Therapeutics, Galway, Ireland  
<sup>6</sup>Cancer Therapy and Research Center, Hematology/Oncology, 7979 Wurzbach Road, SA, Texas 78230, USA  
 E-mail: swordsr@uthscsa.edu



# Appendix C

MYELOID NEOPLASIA

## Brief report

### Inhibition of NEDD8-activating enzyme: a novel approach for the treatment of acute myeloid leukemia

Ronan T. Swords,<sup>1</sup> Kevin R. Kelly,<sup>1</sup> Peter G. Smith,<sup>2</sup> James J. Garnsey,<sup>2</sup> Devalingam Mahalingam,<sup>1</sup> Ernest Medina,<sup>1</sup> Kelli Oberheu,<sup>1</sup> Swaminathan Padmanabhan,<sup>1</sup> Michael O'Dwyer,<sup>3</sup> Steffan T. Nawrocki,<sup>1</sup> Francis J. Giles,<sup>1</sup> and Jennifer S. Carew<sup>1</sup>

<sup>1</sup>Institute for Drug Development, Cancer Therapy and Research Center at the University of Texas Health Science Center at San Antonio; <sup>2</sup>Millennium Pharmaceuticals, Cambridge, MA; and <sup>3</sup>University College Hospital, Galway, Ireland

NEDD8 activating enzyme (NAE) has been identified as an essential regulator of the NEDD8 conjugation pathway, which controls the degradation of many proteins with important roles in cell-cycle progression, DNA damage, and stress responses. Here we report that MLN4924, a novel inhibitor of NAE, has potent activity in acute myeloid leukemia (AML) models.

MLN4924 induced cell death in AML cell lines and primary patient specimens independent of Fms-like tyrosine kinase 3 expression and stromal-mediated survival signaling and led to the stabilization of key NAE targets, inhibition of nuclear factor- $\kappa$ B activity, DNA damage, and reactive oxygen species generation. Disruption of cellular redox status was shown to

be a key event in MLN4924-induced apoptosis. Administration of MLN4924 to mice bearing AML xenografts led to stable disease regression and inhibition of NEDD8-ligated cullins. Our findings indicate that MLN4924 is a highly promising novel agent that has advanced into clinical trials for the treatment of AML. (*Blood*. 2010;115(18):3796-3800)

## Introduction

Acute myeloid leukemia (AML) is a disease of the elderly, and the majority of newly diagnosed patients will be older than 60 years of age.<sup>1</sup> Complete remission occurs in up to half of these patients. However, relapse is generally inevitable and prognosis is dismal. Although novel therapeutic approaches have the potential to improve outcomes for all patients with AML, more effective treatment strategies are urgently required for elderly patients who currently have a much poorer prognosis than younger patients with this disease. Preexisting myelodysplasia, unfavorable cytogenetics, treatment-related AML, and multidrug resistance are all more common in older patients.<sup>2</sup> In addition, coexisting morbidities limit therapeutic options for many of these patients. Moreover, no standard induction approach exists for this group in part because of their poor representation in clinical studies. A recent study revealed that induction with low-dose cytarabine offered a survival advantage over supportive care for patients with good or intermediate prognosis cytogenetics (25% vs 10%,  $P = .004$ ). However, no complete remissions were observed for those with poorer performance status and/or unfavorable cytogenetics on this study, thus highlighting the need for new therapies.<sup>3</sup>

The ubiquitin-proteasome system is responsible for the timed destruction of most intracellular proteins. NEDD8-activating enzyme (NAE) has been identified as an essential controller of the Nedd8 conjugation pathway, which regulates the activity of the cullin-dependent E3 ligases.<sup>4</sup> The cullins control the ubiquitination and subsequent degradation of many proteins with important roles in cell-cycle progression, DNA damage, stress responses, and signal transduction.<sup>5-7</sup> Considering that NEDD8 controls the homeostasis of proteins vitally important for the

survival of AML cells,<sup>5-7</sup> we evaluated the preclinical antileukemic activity of MLN4924, a novel first-in-class small molecule inhibitor of NAE.<sup>8</sup>

## Methods

### Cells and cell culture

HL-60 and HS-5 cells were obtained from ATCC. MV4-11, MOLM-13, and PL-21 cells were obtained from DSMZ. Primary human AML cells were isolated from the bone marrow of AML patients after obtaining informed consent in accordance with the Declaration of Helsinki. The University of Texas Health Science Center institutional review board approved the collection of peripheral blood and bone marrow specimens.

### Chemicals and reagents

Reagents were obtained as follows: MLN4924 (Millennium Pharmaceuticals), antitubulin, antiactive caspase-3, antiphospho- and total I $\kappa$ B $\alpha$ , anti-CDT-1, anti-FLIP, anti-BCL-2, anti-BCL-xL, antiphospho- and total Chk1 antibodies (Cell Signaling), anti-NRF-2 antibody (Santa Cruz Biotechnology), and anti- $\beta$  tubulin (Sigma-Aldrich).

### Cell viability assay

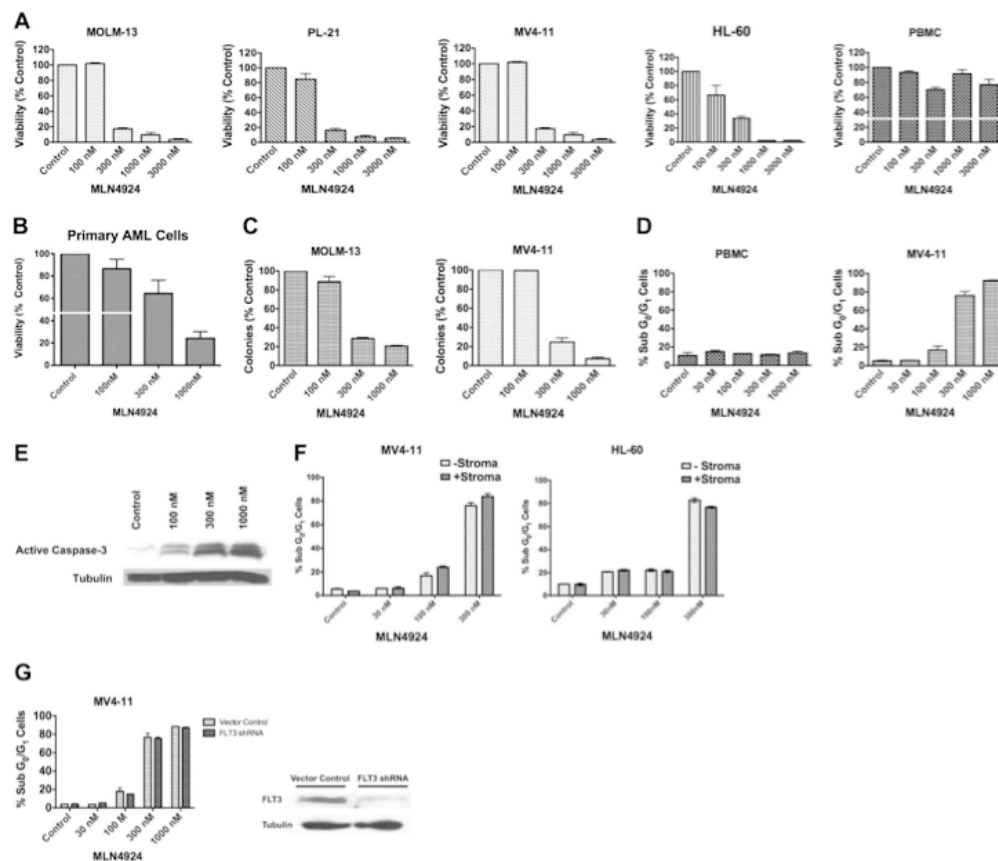
Cells were plated in triplicate and treated with defined concentrations of MLN4924 for 72 hours. Viable cells were quantified using ATPase according to the manufacturer's instructions (PerkinElmer Life and Analytical Sciences).<sup>8</sup>

Submitted November 17, 2009; accepted February 4, 2010. Prepublished online as *Blood* First Edition paper, March 4, 2010; DOI 10.1182/blood-2009-11-254862.

The online version of this article contains a data supplement.

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**Figure 1. MLN4924 is highly active in preclinical AML models and primary AML cells.** (A) MLN4924 potently reduces the viability of AML cells. MOLM-13, PL-21, MV4-11, and HL-60 cell lines and PBMCs from healthy donors were treated with the indicated concentrations of MLN4924 for 72 hours. Viability was assessed by the ATP-Lite assay. Results shown represent the mean of 3 experiments. Error bars represent SD. (B) MLN4924 has activity in primary AML cells. Primary cells were obtained from 5 AML patients. Of these patients, 1 had therapy-related AML and another had primary refractory AML indicating aggressive clinical disease. Primary cells were treated with MLN4924 for 72 hours. Viability was assessed by the ATP-Lite assay. (C) Dose-dependent inhibition of clonogenic survival after treatment with MLN4924. MOLM-13 and MV4-11 human AML cells were treated with the indicated concentrations of MLN4924 for 24 hours. Drug was washed away, cells were seeded in Methocult, and colonies were scored on day 10.  $n = 3$ ; bars represent the mean  $\pm$  SD. (D) Quantification of drug-induced apoptosis. MV4-11 cells and PBMCs from healthy donors were treated with the indicated concentrations of MLN4924 for 48 hours. Percentages of cells with subdiploid DNA were determined by PI/FACS.  $n = 3$ ; bars represent the mean  $\pm$  SD. (E) Activation of apoptosis after treatment with MLN4924. Cells were treated with the indicated concentrations of MLN4924 for 24 hours. Protein lysates were subjected to SDS-PAGE, blotted, and probed with an active caspase-3 specific antibody. Tubulin documented equal loading. (F) Effect of stromal coculture on the proapoptotic activity of MLN4924. MV4-11 and HL-60 cells were treated with the indicated concentrations of MLN4924 for 48 hours in the presence and absence of HS-5 bone marrow stromal cells. Percentages of apoptotic cells were determined by PI/FACS.  $n = 3$ ; bars represent the mean  $\pm$  SD. (G) Impact of FLT3 expression on the sensitivity of AML cells to MLN4924. MV4-11 cells with and without stable shRNA-mediated knockdown of FLT3 expression were treated with the indicated concentrations of MLN4924 for 48 hours. Percentages of apoptotic cells were determined by PI/FACS.  $n = 3$ ; bars represent the mean  $\pm$  SD. Immunoblotting was used to confirm knockdown of FLT3 expression. (Figure 1 and legend continue on next page).

#### Analysis of drug-induced apoptosis

Apoptosis was evaluated by propidium iodide/fluorescence-activated cell sorter (PI/FACS) analysis of sub-G<sub>2</sub>/G<sub>1</sub> DNA content as previously described.<sup>9</sup>

#### Colony assays

AML cells were treated with MLN4924 for 24 hours, seeded in Methocult, and colonies were scored as previously described.<sup>10</sup>

#### Immunoblot analyses

Protein extracts were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) electrophoresis and transferred to nitrocellulose

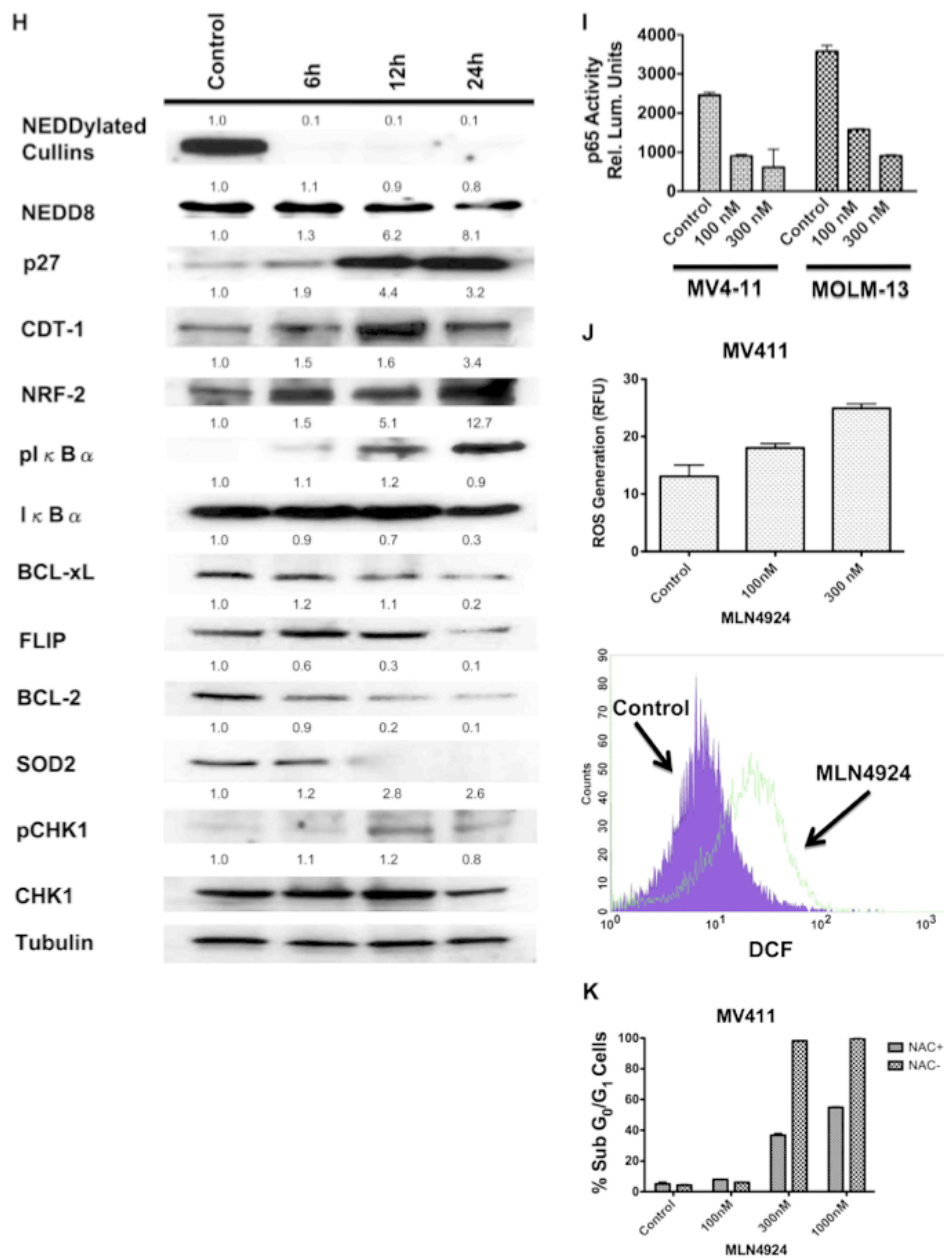
membranes as described previously.<sup>9</sup>  $\beta$ -Tubulin documented equal loading.

#### Stable knockdown of FLT3 expression

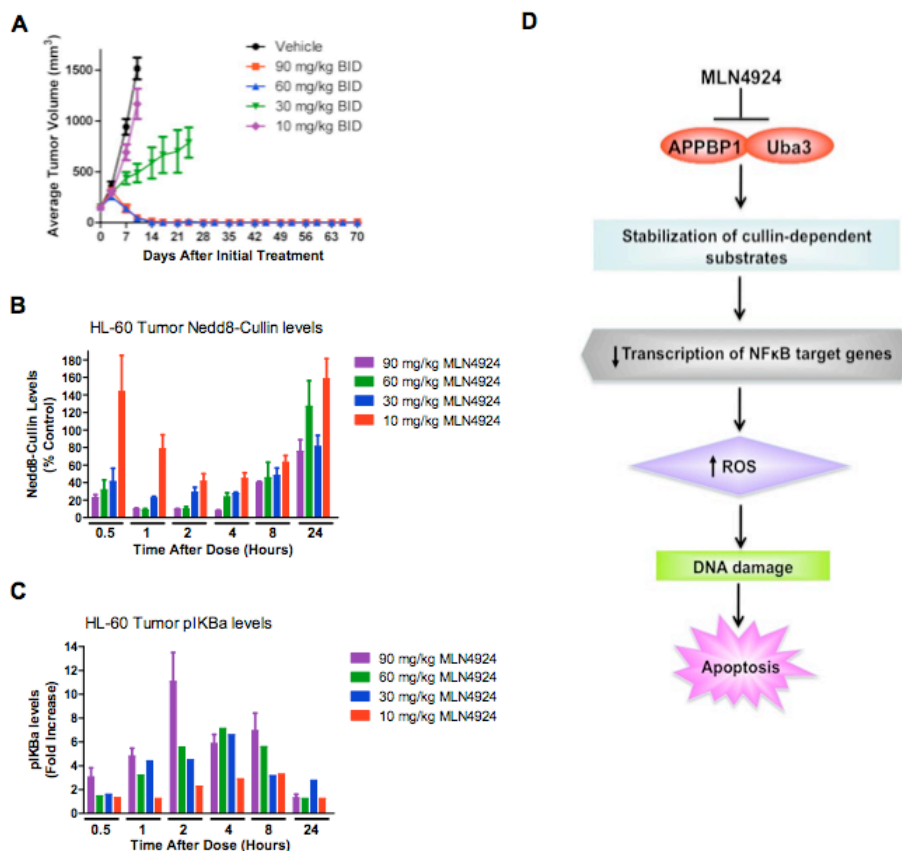
SMARTVector lentiviral particles containing empty vector (control) and Fms-like tyrosine kinase 3 (FLT3) shRNA were used to infect MV4-11 cells according to the manufacturer's instructions (Dharmacon RNA Technologies).

#### Quantification of NF- $\kappa$ B DNA-binding activity

DNA binding was quantified using the chemiluminescent nuclear factor- $\kappa$ B (NF- $\kappa$ B) p65 transcription factor kit according to the manufacturer's directions (Pierce Protein Research Products).



**Figure 1 (continued).** (H) Effects of MLN4924 on NEDDylated substrates and downstream effectors. Cells were treated with MLN4924 for 24 hours, lysed, subjected to SDS-PAGE, and probed with NEDD8, p27, CDT-1, NRF-2, pCHK1, total CHK1, pI $\kappa$ B $\alpha$ , total I $\kappa$ B $\alpha$ , BCL-2, BCL-xL, FLIP, and SOD2-specific antibodies. Tubulin documented equal loading. Densitometry analysis was carried out by quantifying the band density for each protein relative to the band density of tubulin using an Alpha Innotech FluorChem HD2 gel documentation system (Alpha Innotech). The densitometry values for all controls were normalized to 1.0. Densitometry values are indicated above each band and reflect the ratio of the change in protein expression from control levels for each respective protein. (I) Effects of MLN4924 on NF $\kappa$ B (p65) DNA-binding activity. Cells were treated with MLN4924 as indicated for 24 hours. Relative p65 DNA-binding activity was quantified using a chemiluminescent detection method. (J) Quantification of MLN4924-induced ROS generation. MV411 cells were treated with MLN4924 for 12 hours, and ROS production was evaluated by staining with dichlorofluorescein (DCF) followed by FACS analysis.  $n = 3$ ; bars represent the mean  $\pm$  SD. (K) Impact of antioxidant treatment on MLN4924-induced apoptosis. Drug-induced apoptosis was quantified after 48 hours of exposure to MLN4924 in the presence and absence of the antioxidant NAC.  $n = 3$ ; bars represent the mean  $\pm$  SD.



**Figure 2.** MLN4924 induces stable disease regression in an AML xenograft model. (A) Administration of MLN4924 to mice bearing HL-60 xenografts leads to a dose-dependent reduction in disease burden. Mice received vehicle control, 10, 30, 60, or 90 mg/kg MLN4924 twice a day for 21 days. Tumor volume was measured by calipers.  $n = 10$  per group; bars represent the mean  $\pm$  SD. (B) Effect of MLN4924 treatment on NEDDylation of cullins in vivo. Mice were administered a single dose of MLN4924, and the levels of NEDDylated cullins were quantified at the indicated time points ( $n = 3$ ). (C) Dose-dependent stabilization of phospho-IκBα after treatment with MLN4924. Mice were given a single dose of MLN4924, and the levels of phospho-IκBα were quantified at the indicated time points after drug administration. (D) Schematic representation of the proposed mechanism of MLN4924-induced apoptosis in AML cells. MLN4924 inhibits the activity of NAE, leading to the abrogation of cullin NEDDylation, the stabilization of NEDD8-regulated substrates, increased ROS production, and DNA damage, which culminates in apoptosis.

#### In vivo evaluation of MLN4924

HL-60 human leukemia cells were injected into the flanks of nude mice. After tumor growth reached 150 mm<sup>3</sup>, mice were randomly assigned to receive MLN4924 10, 30, 60, or 90 mg/kg twice a day ( $n = 10$  per group), vehicle control ( $n = 10$ ) for 21 days. Tumor growth and animal toxicity were assessed as previously described.<sup>11</sup> The University of Texas Health Science Center institutional animal care and use committee approved the mouse studies.

#### Results and discussion

We first assessed the effects of MLN4924 treatment on the viability of AML cells. Nanomolar concentrations of MLN4924 (mean 50% inhibitory concentration = 211 nM) selectively and potently inhibited the in vitro growth and survival of MOLM-13, PL-21, MV4-11, and HL-60 cells and primary AML cells from patients with different clinical and molecular features compared with

peripheral blood mononuclear cells (PBMCs) from healthy donors (Figure 1A-B; supplemental Table 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). MLN4924 treatment also dramatically disrupted the ability of AML cells to form colonies (Figure 1C) and led to a dose-dependent induction of apoptosis as evidenced by sub-G<sub>0</sub>/G<sub>1</sub> DNA content and the processing of caspase-3 to its active form (Figure 1D-E).

Drug resistance continues to be a major multifaceted problem that limits successful clinical outcomes for patients with AML. Stromal-mediated survival signaling has been proposed as an important mechanism of resistance to many classes of anticancer agents.<sup>12</sup> Considering this, we investigated the impact of stromal interactions on the sensitivity of AML cells to MLN4924. Coculturing MV4-11 and HL-60 AML cells with HS-5 human stromal cells did not significantly impact the proapoptotic activity of MLN4924 (Figure 1F). This indicates that MLN4924 can overcome the survival advantage provided by stroma (supplemental Figure 1).



Similarly, MV4-11 cells with and without stable shRNA-mediated knockdown of FLT3 expression responded equally to treatment with MLN4924. This suggests that MLN4924 may be an effective agent for patients with FLT3 internal tandem duplication and/or activating mutations in FLT3, which are typically associated with inferior outcomes to conventional induction therapy (Figure 1G).

We next investigated the effects of MLN4924 on the NEDDylation of cullins and the expression of cullin-dependent substrates in AML cells. Inhibition of NAE activity with MLN4924 produced a time-dependent decrease in the levels of NEDDylated cullins (Figure 1H), leading to stabilization of cullin-dependent substrates (p27, CDT-1, NRF-2, and phospho-I $\kappa$ B $\alpha$ ) and activation of the DNA damage sensor, CHK1 (Figure 1H).

The NF- $\kappa$ B transcription factors are constitutively active in many human cancers and regulate the transcription of several genes with fundamental roles in survival signaling and drug resistance.<sup>13-15</sup> One potential underlying cause of constitutive NF- $\kappa$ B activity is the inappropriate degradation of its inhibitor, I $\kappa$ B $\alpha$ .<sup>13,16</sup> We hypothesized that MLN4924-mediated disruption of the NEDDylation of I $\kappa$ B $\alpha$  would lead to the inhibition of NF- $\kappa$ B transcriptional activity. Treatment with MLN4924 resulted in a time-dependent decrease in the expression of the NF- $\kappa$ B targets BCL-2, BCL-xL, FLIP, and superoxide dismutase 2 (SOD2). Accordingly, MLN4924 treatment also resulted in a significant reduction in the DNA-binding activity of the p65 subunit of NF- $\kappa$ B (Figure 1I).

Many types of malignant cells generate significantly higher levels of reactive oxygen species (ROS) than their normal counterparts. This phenomenon can be therapeutically exploited to selectively kill cancer cells using agents that induce further ROS stress, which culminates in the induction of apoptosis.<sup>17</sup> We hypothesized that the significant decrease in the expression of the antioxidant enzyme SOD2 induced by MLN4924 may result in increased ROS generation. Treatment with MLN4924 for 12 hours led to a significant, dose-dependent increase in ROS generation (Figure 1J). To test the contribution of MLN4924-induced ROS production to its anticancer mechanism of action, we evaluated the proapoptotic effects of MLN4924 in the presence and absence of the antioxidant N-acetylcysteine (NAC). NAC treatment significantly blunted the degree of apoptosis stimulated by MLN4924, indicating that ROS production is an important event in MLN4924-induced cell death.

The in vivo anticancer activity of MLN4924 was evaluated by administering MLN4924 or vehicle control to mice implanted with HL-60 xenografts. MLN4924 treatment led to a dose-dependent decrease in disease burden, and 10 of 10 animals in the groups treated with 60 and 90 mg/kg experienced stable regressions (Figure 2A). Analysis of specimens collected from animals after administration of a single dose of MLN4924 demonstrated in vivo inhibition of cullin NEDDylation and accumulation of phospho-I $\kappa$ B $\alpha$  (Figure 2B-C). Our collective findings indicate that targeting NAE activity is a novel and highly effective approach for the treatment of AML that warrants further investigation in a clinical trial.

## Acknowledgments

This work was supported by LeukemiaTexas (J.S.C.), the Owens Biomedical Research Foundation (J.S.C.), and the AT&T Foundation (F.J.G.).

## Authorship

Contribution: R.T.S. was involved in all aspects of the study, including experimental design, performing research, data analysis, and manuscript preparation; K.R.K., P.G.S., and J.J.G. provided intellectual input regarding experimental design and data interpretation, performed research, and were involved in the preparation of the manuscript; S.T.N. provided intellectual input and performed research; D.M., E.M., and K.O. performed research and contributed to data analysis; S.P., M.O., S.T.N., and F.J.G. provided intellectual input regarding experimental design, data interpretation, and manuscript preparation; and J.S.C. directed the study and was involved in all aspects of experimental design, data analysis/interpretation, and manuscript preparation.

Conflict-of-interest disclosure: P.G.S. and J.J.G. are employees of Millennium Pharmaceuticals. The remaining authors declare no competing financial interests.

Correspondence: Jennifer S. Carew, Institute for Drug Development, Cancer Therapy and Research Center at University of Texas Health Science Center, 14960 Omicron Dr, San Antonio, TX 78245; e-mail: carew@uthscsa.edu.

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# Appendix D



<b>Start</b>
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## 658 The Novel, Investigational NEDD8-Activating Enzyme Inhibitor MLN4924 In Adult Patients with Acute Myeloid Leukemia (AML) or High-Grade Myelodysplastic Syndromes (MDS): A Phase 1 Study

**Program:** Oral and Poster Abstracts

**Type:** Oral

**Session:** Acute Myeloid Leukemia - Therapy, excluding Transplantation: Novel Therapeutics

**Monday, December 6, 2010: 5:15 PM**

311 ABCD (Orange County Convention Center)

**Ronan T Swords, MD, MRCPI, FRCPath<sup>1</sup>\*, Harry P Erba, MD, PhD<sup>2\*</sup>, Daniel J DeAngelo, MD, PhD<sup>3\*</sup>, Peter G Smith, PhD<sup>4</sup>, Michael D Pickard, MA<sup>5\*</sup>, Bruce J. Dezube, MD<sup>6</sup>, Francis J. Giles, MB, MD, FRCPI, FRCPath<sup>7</sup> and Bruno C. Medeiros, MD<sup>8\*</sup>**

<sup>1</sup>Institute for Drug Development, Cancer Therapy and Research Center at the University of Texas Health Science Center, San Antonio, TX

<sup>2</sup>Hematology/Oncology, University of Michigan Comprehensive Cancer Center, Ann Arbor, MI

<sup>3</sup>Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA

<sup>4</sup>Discovery, Millennium Pharmaceuticals, Inc., Cambridge, MA

<sup>5</sup>Biostatistics, Millennium Pharmaceuticals, Inc., Cambridge, MA

<sup>6</sup>Oncology Clinical Research, Millennium Pharmaceuticals, Inc., Cambridge, MA

<sup>7</sup>Medicine, Cancer Therapy and Research Center, CTTC at The UT Health Science Center, The Institute for Drug Development, San Antonio, TX

<sup>8</sup>Division of Hematology, Stanford Comprehensive Cancer Center, Stanford, CA

**Background:** NEDD8-activating enzyme (NAE) regulates the NEDD8 conjugation pathway, and is required for the activity of the cullin-RING E3 ligases (CRLs). CRLs control the timed degradation of several substrates involved in cell-cycle regulation, signal transduction, DNA replication, and stress response, including proteins important for the survival of AML cells. We evaluated the preclinical anti-leukemic activity of MLN4924, a novel, investigational, first-in-class small molecule inhibitor of NAE, and based on the activity of MLN4924 in preclinical AML models (Swords RT et al, Blood 2010) we conducted a phase 1 study to evaluate the safety and tolerability of this agent in patients with AML and advanced MDS. **Methods:** The primary objectives of this study were to evaluate the safety and tolerability of MLN4924, to establish the maximum tolerated dose (MTD), and to determine the recommended phase 2 dose of MLN4924 in patients with AML and high-grade MDS. Secondary objectives included a preliminary assessment of efficacy, and analysis of pharmacokinetics and pharmacodynamics (via NAE-regulated proteins in peripheral blood mononuclear cells). Patients aged  $\geq 18$  years, with ECOG performance status 0-2, who had AML or high-grade MDS, and who were not candidates for potentially curative therapy, were eligible. MLN4924 was administered as a 60-minute IV infusion on days 1, 3, and 5 of a 21-day cycle for up to 12 months or until documented disease progression. Dose escalation was commenced at 25 mg/m<sup>2</sup> and proceeded using a standard '3+3' escalation method until the MTD was established. Response assessment was based on recently published guidelines (Döhner H et al, Blood 2010) and adverse events (AEs) were graded per National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events v3.0 (NCI Cancer Therapy Evaluation Program, 2006). **Results:** To date, 15 patients (9 males, 6 females; 14 AML, 1 high-grade MDS) have been enrolled and treated, including 3, 4, 3, 3, and 2 at dose levels of 25, 33, 44, 59, and 78 mg/m<sup>2</sup>, respectively. Median age was 62.3 years (range 29.3-84.0 years). By cytogenetics, 1 (7%), 5 (33%), and 7 (47%) patients had good-, intermediate-, and poor-risk disease (not available in 2). Prior antineoplastic therapies included cytarabine (n=7), azacitidine, daunorubicin (n=3 each), decitabine, etoposide, gemtuzumab, idarubicin, and mitoxantrone (n=2 each). To date, 3 patients have received  $\geq 8$  cycles; 6 remain on treatment. Two dose-limiting toxicities have been reported at the 78 mg/m<sup>2</sup> dose level: one patient with multi-organ failure in Cycle 2, and one with reversible elevation of alanine aminotransferase in Cycle 1. The most common AEs were pneumonia (n=6), atelectasis, constipation, diarrhea, and febrile neutropenia (each n=4); most common grade  $\geq 3$  AEs were febrile neutropenia (n=4), elevated aspartate aminotransferase, and pneumonia (each n=3). Three patients have achieved a complete response (CR) to date. A 29-year-old woman with relapsed AML following allogeneic stem cell transplantation achieved a CR after cycle 1 at 25 mg/m<sup>2</sup> before developing progressive disease at an extramedullary site during cycle 8. An 82-year-old man with history of high-risk MDS, which was unresponsive to azacitidine, that evolved into AML had a partial response in cycle 8 and a CR with incomplete recovery of blood counts (CRI) in cycle 10 at 33 mg/m<sup>2</sup>; the patient is currently in cycle 12 and has become transfusion-independent. A 71-year-old man with de-novo AML refractory to standard cytarabine plus daunorubicin induction achieved a CRI during cycle 1 at 44 mg/m<sup>2</sup>; although this was not maintained, the patient continued to benefit from treatment and is currently in cycle 11 with reduced transfusion dependence. Pharmacodynamic data are available for 9 patients; 7 show evidence of target inhibition in peripheral blood by changes in NAE-regulated proteins. **Conclusion:** The preliminary findings of this study indicate that the novel mechanism of action of MLN4924 through NAE inhibition results in observed activity in patients with relapsed or refractory AML, and suggest the successful translation of preclinical research in AML models into the clinic. Enrollment continues in expanded cohorts of AML and MDS patients at 59 mg/m<sup>2</sup>. Updated efficacy and safety data will be presented, together with data on MLN4924 pharmacokinetics and pharmacodynamics.

**Disclosures:** **Off Label Use:** Investigational agent in clinical development for the treatment of acute myeloid leukemia or myelodysplastic syndromes. **Erba:** Millennium Pharmaceuticals, Inc.: Research Funding. **DeAngelo:** Deminimus: Consultancy. **Smith:** Millennium Pharmaceuticals, Inc.: Employment. **Pickard:** Millennium Pharmaceuticals, Inc.: Employment. **Dezube:** Millennium Pharmaceuticals, Inc.: Employment, Equity Ownership. **Giles:** Millennium Pharmaceuticals, Inc.: Research Funding. **Medeiros:** Millennium Pharmaceuticals, Inc.: Consultancy, Research Funding.

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