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It is entitled:
IRAK family kinases as therapeutic targets for Myelodysplastic Syndrome and Acute Myeloid Leukemia

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IRAQ family kinases as therapeutic targets for Myelodysplastic Syndrome and Acute Myeloid Leukemia

A dissertation submitted to the Graduate School of the University of Cincinnati in partial fulfillment of the requirements to the degree of Doctor of Philosophy

in the Department of Cancer and Cell Biology of the College of Medicine

by

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Abstract

Innate immune signaling has an essential role in inflammation, and the dysregulation of signaling components within this pathway is increasingly being recognized as a mediator in cancer initiation and progression. The innate immune system is an evolutionarily conserved pathogen pattern recognition apparatus, which defends the host in a non-specific manner. Pathogens and cytokines signal to immune cells through the toll-like receptor (TLR) and interleukin-1 receptor (IL1R) superfamily. In order to mediate an inflammatory response, TLRs and IL1R require interleukin-1 associated receptor kinases (IRAKs). Herein, we demonstrate that IRAK1 is activated and overexpressed in Myelodysplastic Syndrome (MDS) and Acute Myeloid Leukemia (AML); two closely related hematologic malignancies. Furthermore, pharmacological (IRAK-Inh) and RNAi-mediated inhibition of IRAK1 is effective in eliminating disease-propagating cells. Integrated gene expression analysis revealed compensatory BCL2 upregulation following small-molecule IRAK1 inhibition. This proved to be a drugable vulnerability, as BCL2 inhibition potently synergized with IRAK-Inh to induce rapid cell death, even in IRAK-Inh-refractory cell lines. Importantly, suppression of IRAK1 signaling, through either RNAi or small-molecule inhibition, is tolerated in normal CD34+ cells, suggesting a potential therapeutic window for MDS and AML patients. To examine the effect of cancer-modifying therapies, like IRAK inhibition, we developed a novel xenograft model, utilizing an MDS-derived patient cell line, MDSL. Immunocompromised animals receiving MDSL xenografts developed progressive anemia and thrombocytopenia, thus recapitulating clinical features of the disease. These mice displayed rapid morbidity resulting from MDSL engraftment in bone marrow, spleen and peripheral blood. In this setting, both RNAi-mediated and small-molecule IRAK1 inhibition was effective in reducing MDSL cell burden, and provided a significant survival benefit. To broaden our findings, we examined the effect of IRAK inhibition in the context of FLT3-mutated AML. Mutations in the type III receptor tyrosine kinase, FLT3, occur in approximately 30% of AML patients and lead to constitutive downstream signaling activation,
thus FLT3 is an attractive molecular-target. Herein we report the structure and activity of several dual IRAK and FLT3 small-molecule kinase inhibitors, which exhibit potent cytotoxicity against D835-mutant FLT3 and FLT3-ITD AML cells. Three of our tool compounds exhibit 4- to 1300-fold greater cellular activity than quizartinib, a FLT3 inhibitor undergoing clinical investigation. Thus combining FLT3 and IRAK pathway inhibition appears to be a promising therapeutic strategy for mutant-FLT3 AML. In sum, our findings reveal the dependency of MDS and AML on TLR/IL1R signaling, and validate IRAK kinases as important therapeutic targets.
Preface

The work presented in this dissertation has been previously published in, or is in preparation for, the following peer-reviewed journals:

Acknowledgements

I’m privileged to have received the guidance and mentorship of many talented and experienced scientists during my tenure at the University of Cincinnati College of Medicine and Cincinnati Children’s Hospital Medical Center. Firstly, I would like to thank my Thesis Committee Chair, Dr. Daniel Starczynowski for his guidance and support. The countless discussions we held were instrumental in molding my ability to effectively articulate science. Ultimately, Dr. Starczynowski’s steadfast belief in my abilities helped me reach ever farther and higher. A short rotation spent in Dr. Jim Mulloy’s lab proved to be a truly invaluable experience and led to an understanding in vivo cancer modeling. During this time I met Mark Wunderlich, who has remained a close friend, and an integral collaborator with a wealth of precious technical and experimental knowledge. Dr. Lee Grimes has been an ardent supporter and at key times during my graduate career has provided me with a vital optimism. Finally, I thank the members of my Thesis Committee for dedicating time to offer direction and critical appraisal of my work.
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Chapter 1: Targeting IRAK signaling in cancer

Selected sections of this Chapter are in preparation for publication:


Development of kinase inhibitors for cancer

Over several decades, careful genetic and biochemical characterization has revealed roles for many of the 530 protein kinases in cellular proliferation, survival and migration. Not surprisingly, the deregulation of protein kinases, through chromosomal rearrangements, mutations, and/or gene amplification drives tumorigenesis (1-6). Approximately half of all genes directly linked to the induction and maintenance of cancer encode protein kinases (7-10). Thus protein kinases represent compelling targets for anti-cancer drug development.

Initial efforts to develop small-molecule inhibitors against this protein class were met with significant skepticism because all kinases share a highly conserved catalytic domain. The kinase domain has two lobes – an N-terminal lobe rich in β-sheets, and an alpha-helical C-terminal domain. The two catalytic lobes are joined by a hinge region, which spans the ATP-binding site. Many scientists believed the well-conserved ATP-binding site of kinases would prevent medicinal chemists from generating high-specificity inhibitors. Despite this high degree of conservation, highly selective small molecules with appropriate pharmaceutical properties have been developed against a number of kinases (11).

To date, there have been 24 unique FDA-approved kinase inhibitors indicated for the treatment of cancer (Table 1). Perhaps surprisingly, these registered agents only target a small portion of the kinome. Unfortunately, the current pipeline development strategy used by much of the pharmaceutical industry has led to a continued focus on familiar drug targets. For example, the enormous commercial success of imatinib has fueled further development of 2nd and 3rd generation BCR-ABL inhibitors capable of overcoming imatinib-resistant mutations (12). Targets
such as the phosphoinositide-3 kinases (PI3Ks), mTOR, AKT and cell-cycle regulating kinases have garnered recent significant investment by biotechnology and pharmaceutical companies, with a number of inhibitors currently undergoing clinical investigation (13-21).

Since, at present, only a small fraction of the kinome can be targeted by selective and potent small-molecule inhibitors, there is a desire to foster new strategies for the discovery and optimization of inhibitors with novel kinase targets. New ATP site-targeted ligands are being developed through the combination of analogue synthesis, structure-based/computational design (22-25), and fragment-based assembly (26, 27). Another challenge facing this drug class is the emergence of resistance to kinase inhibition (22). Clinical studies have revealed that kinase inhibition can result in resistance due to target mutation and/or amplification (28-31), or through compensatory mechanisms allowing the cancer cell to bypass inhibition through overexpression of alternative oncogenes (32). This has compelled the biotechnology and pharmaceutical industry to initiate the development of inhibitors capable of circumventing target-related drug resistance (12, 33, 34). It appears possible to reduce, and in some instances, eliminate on-target resistance mechanisms through the use of allosteric inhibitors, however overcoming compensatory resistance may prove to be more challenging.

Myelodysplastic syndrome (MDS)

MDS are clonal hematopoietic stem cell (HSC) disorders in which genetic and/or epigenetic alterations compromise the normal function of hematopoietic stem and progenitors cells (HSPC), resulting in blood cytopenias and marrow cell dysplasia (35).

Approximately 30% of MDS patients will also progress to acute myeloid leukemia (AML) upon acquisition of additional mutations in the HSPC compartment (36). Moreover, MDS patients who progress to AML experience lower therapeutic response rates than patients with de novo AML. The underlying molecular, genetic, and phenotypic heterogeneity associated with MDS presents a challenge to the accurate diagnosis, prognosis, and treatment of this disease.
The World Health Organization (WHO) has classified MDS into eight subtypes based on morphology, immunophenotype, genetics and clinical features (37). MDS subtypes include refractory anemia (RA), refractory anemia with ringed sideroblasts (RARS), refractory anemia with multilineage dysplasia (RCMD), refractory anemia with multilineage dysplasia and ringed sideroblasts (RCMD-RS), refractory anemia with excess blasts-1 (RAEB-1), refractory anemia with excess blasts-2 (RAEB-2), MDS unclassified (MDS-U), and MDS associated with isolated deletion chromosome 5q (del(5q)) (known as 5q- syndrome). Despite its value for diagnostic categorization of patients with MDS, the prognostic limitations of the WHO classification are evinced through widely variable clinical outcomes within the WHO subtypes. Most recently, the Revised International Scoring System (IPSS-R) was developed as an additional risk-based stratification system. In this analysis, cytogenetic, morphologic and clinical data were combined and statistically weighted from a large group of MDS cases that had been previously reported in prognostic studies. Risk assessment is divided into four categories: low-risk, intermediate-1 risk, intermediate-2 risk, and high-risk.

Regardless of subtype or risk category, recent evidence suggests that the HSC is the disease-initiating cell. MDS is thought to arise from a CD34+ HSC that has acquired genetic and/or epigenetic abnormalities (38, 39). It is thought that the acquired mutations result in the clonal advantage of a defective HSC that is incapable of supporting normal blood cell production (40). In some cases, subsequent additional mutations are thought to impair normal differentiation and result in accumulation of highly proliferative leukemic blasts, thus promoting transformation to AML.

Clinicians deploy the IPSS-R to determine appropriate therapeutic options for MDS patients. Additionally, the National Comprehensive Cancer Network (NCCN) guidelines recommend that all patients receive supportive care, and be subsequently divided into either a low- or high-risk disease category using IPSS-R criteria (41). A small proportion of patients are eligible for curative hematopoietic stem cell transplantation (HSCT), although this option is
fraught with high treatment-related mortality and other complications (42). Instead, most patients will receive one or more of the following: growth factors (43), high-intensity induction therapy, immunomodulatory drugs (44), and/or hypomethylating agents. At present, azacitidine is the only medicine capable to yield a significant, albeit small, benefit in overall survival when compared to standard care (24.5 vs 15.0 months, respectively) (45). Not surprisingly, recent work has demonstrated that azacitidine fails to eradicate the disease-initiating HSC in MDS patients (46). Therefore, there is an unmet need to develop medicines that can effectively target the MDS-initiating HSC. Insufficient knowledge regarding the basic biology of MDS remains an impediment to the development of effective treatment strategies, capable of providing durable, long-term responses.

**IRAQ family kinases**

The interleukin-1 receptor associated kinases (IRAQs) are key mediators of toll-like receptor (TLR) and interleukin-1 receptor (IL1R) signaling processes. TLR/IL1R-mediated signaling has been shown to control diverse cellular processes including inflammation, apoptosis, and cellular differentiation. The variegation of TLR/IL1R signaling is achieved through differential recruitment of adaptor molecules such as MyD88, Mal/TIRAP, TRIF and TRAM. These adaptors function in the subsequent recruitment and activation of IRAQ family kinases. Four IRAK genes exist in the human genome (IRAK1, IRAK2, IRAK3 and IRAK4), and studies, particularly with transgenic mice, have revealed distinct, non-redundant biological roles (47-51). All IRAK proteins share a similar domain structure, including a N-terminal death domain important for dimerization and MyD88 interaction, a ProST domain, and a kinase and/or pseudokinase domain, however only IRAK1, 2, and 3 have a C-terminal domain important for TRAF6 activation (Figure 1B). Further biochemical work has revealed differential post-translational modification, cellular localization, and regulation of IRAK family members (52-57). Although IRAKs are categorized as serine/threonine protein kinases, only IRAK1 and IRAK4
exhibit kinase activity (58, 59). Human epidemiological studies, as well as transgenic mouse models, have linked genetic variations in IRAK genes to a diverse collection of diseases including infection, sepsis, atherosclerosis, autoimmunity, systemic lupus erythematosus, and cancer (47, 60-66).

**IRAK1**

IRAK1, the first member of the IRAK family to be discovered, was identified through biochemical isolation of an IL1 dependent kinase activity which co-immunoprecipitated with the IL1R (58). Upon IL1R/TLR ligand binding, MyD88 is rapidly recruited to the receptor through its TIR domain. IRAK1 interacts with MyD88 via its death-domain (DD) and undergoes subsequent activation (67-70). The activation and phosphorylation of IRAK1 is a multistep process, but initially requires a critical T209 residue, as mutation of this residue completely disrupts IRAK1 kinase activity (55). IRAK1 is subsequently phosphorylated at key residues within its activation loop and ProST region. MyD88 only binds non-phosphorylated IRAK1 (57), and upon phosphorylation IRAK1 is released from the receptor complex to bind the E3 ubiquitin ligase, TRAF6, to activate NF-κB.

IRAK1 is the substrate of additional covalent modifications, including ubiquitination and sumoylation, which impact function and localization (57, 68, 71). After becoming phosphorylated, IRAK1 can undergo K48-linked ubiquitination and subsequent rapid degradation via the 26S proteasome (57). Additionally, IRAK1 can be modified through K63-linked polyubiquitin (72). This is thought to be an activating mark, as mutation of K63-linked ubiquitin sites on IRAK1 prevents NEMO binding and subsequent NF-κB activation. The proportion of higher molecular weight modified forms of IRAK1 increases upon LPS stimulation (47) and is thought to result from ubiquitination, in addition to hyperphosphorylation of the proline/serine/threonine-rich (ProST) domain (Figure 1.1A). IRAK1 localizes to both the
cytoplasm and nucleus (73, 74), however the modified, higher-molecular weight form is predominantly found in the nuclear fraction, and sumoylation of IRAK1 is necessary for nuclear entry (56). Thus, post-translational modifications of IRAK1 are necessary for regulating diverse functions, including nuclear trafficking, degradation, and kinase activation.

IRAK1-deficient mice have been used to interrogate the role of IRAK1 within IL1/TLR-mediated activation of NF-κB and MAPK signaling pathways (51, 75, 76). IRAK1-/- macrophages display a decrease in LPS-induced IKKβ activation and IL1/LPS-induced NF-κB DNA binding. Additionally, primary mouse embryonic fibroblasts (MEFs) isolated from IRAK1-/- mice displayed reduced IL1-induced p38 and JNK activation. Additionally, these studies have revealed a critical role for IRAK1 in nuclear STAT3 activation and subsequent IL-10 gene expression (47). This is of clinical relevance, since elevation of IL-10 levels is a common phenomenon among atherosclerosis patients, and this coincides with IRAK1 nuclear localization (47).

Depending on cellular context, a kinase-dead IRAK1 mutant can rescue the loss of NF-κB activation observed in IRAK1-deficient cells (77). Indeed both wild-type and kinase dead IRAK1 protein are capable of activating NF-κB transcriptional activity. Recently, a catalytically inactive IRAK1 D359A mutant mouse was reported (78). Bone marrow-derived monocytes (BMDM) from this mouse did not exhibit impairment in the activation of the canonical IKK complex, MAPK activation, or the production of IL6, IL10 and TNF-α mRNA. However, plasmacytoid dendritic cells (pDCs) from IRAK1 D359A mice displayed greatly delayed TLR7- and TLR9-induced IFN-α and IFN-β mRNA production. Thus the catalytic requirement of IRAK1 appears to be context and cell-type specific.
IRAK2

IRAK2 plays a critical role in proximal TLR signaling and in the activation of NF-κB (53). IRAK2 is a necessary component of a multimeric helical MyD88-IRAK4-IRAK2 signaling complex that is formed through death-domain interactions downstream of TLR/IL1R activation (Figure 1.1B) (79). Unlike the other IRAK family members, IRAK2 is capable of interacting with the TLR3 signaling adaptor Mal/TIRAP, and is recruited to TLR3 through death-domain interactions (80). Along with IRAK1, IRAK2 is also important in the formation of polyubiquitin chains associated with TRAF6 signaling (53). Interestingly, IRAK2-deficient mice are more resistant to LPS and CpG-induced septic shock than IRAK1-deficient animals (81). Although IRAK1 and IRAK2 function redundantly in initial TLR signaling responses, IRAK2 plays a critical role in late-phase TLR signaling, namely in cytokine production (81). Mouse knock-in studies have established that the IRAK2-TRAF6 interaction is rate limiting for the late phase cytokine production in BMDMs and pDCs, and that this interaction is critical to sustaining NF-κB signaling during prolonged activation of MyD88 signaling (78).

IRAK3 (IRAK-M)

Human IRAK3 gene expression is restricted to monocytes and macrophages. Although initial studies reported that IRAK3 could activate NF-κB (82), more recent literature has demonstrated the powerful negative regulatory role IRAK3 plays within the context of TLR signaling (Figure 1.1B). IRAK3/-/- macrophages exhibit elevated levels of inflammatory cytokines upon TLR ligand challenge, and IRAK3/-/- mice show a hyper-inflammatory response to bacterial infection (48). Additionally, endotoxin tolerance is significantly reduced in IRAK3/-/- cells, thus IRAK3 regulates TLR signaling and innate immune homeostasis. At the molecular level, IRAK3 exerts negative regulatory effects through preventing: (i) the dissociation of IRAK1 and IRAK4 from MyD88, and (ii) the formation of the IRAK1-TRAF6 signaling complex (48). Recently,
IRAK3 was identified as a regulator of hematopoiesis in a functional zebrafish screen, and thus could potentially play a role in HSC self-renewal and differentiation (83).

**IRAK4**

IRAK4 is the closest homolog to the Drosophila Pelle protein. As the only IRAK member in the fly, Pelle is a signaling mediator of the Toll-Dorsal pathway during embryonic development. Following the engagement of TLR agonists or IL1, IRAK4 is recruited to the protein adaptor MyD88 through death-domain interactions (50, 59). IRAK4, IRAK2 and MyD88 can form a large oligomeric left-handed helical signaling complex, termed the Myddosome (Figure 1.1B) (79, 84). The assembly of this higher-order complex leads to the IRAK4-mediated recruitment and phosphorylation of IRAK1 (59). Interestingly, overexpression of IRAK4 mutants containing truncations within the N-terminal kinase domain can suppress IL1-inducible recruitment of wild-type IRAK4 to the IL1R complex, and prevent association with IRAK1, while enabling sequestration of MyD88 (85). In contrast to IRAK1-deficient mice, IRAK4-/- animals display a severe impairment inflammatory cytokine expression and NF-κB activation upon challenge with TLR ligands or IL1, and are completely resistant to LPS-mediated septic shock (50). Additionally, IL1-induced JNK and p38 activation is completely defective in cells lacking IRAK4.

Studies examining kinase-dead IRAK4 knock-in mice demonstrated the requirement of kinase activity for certain IRAK4-dependent activities (86, 87). Similar to IRAK4-deficient animals, IRAK4 kinase-dead mice were resistant to TLR-induced septic shock (88, 89). Perhaps surprisingly, macrophages from IRAK4 kinase-dead mice were capable of activating NF-κB through IL1, TLR2, TLR4 and TLR7, suggesting kinase dispensable activities of IRAK4 (86, 87). Interestingly, while IL1/TLR-induced NF-κB activation was not greatly impaired in IRAK4 kinase dead knock-in mice, there was severe impairment of IL1/TLR-induced cytokine production and
JNK activation (87-89). Further studies examining IRAK4-deficient human cells reconstituted with kinase dead IRAK4 have revealed redundancies in IRAK4 kinase activity. In human fibroblasts, kinase dead IRAK4 was capable of restoring IL1-induced NF-κB, JNK activation and IL8 gene expression to a similar degree as IRAK4 (90). Thus there may be context-specific redundancies between IRAK kinase activities.

Human IRAK4 deficiency has been described as an autosomal recessive disorder (91, 92). As a result of IRAK4 deficiency, patients suffer from recurrent infections caused by gram-positive pyogenic bacteria such as *Streptococcus pneumoniae* (93-98). Blood cells from these patients fail to generate pro-inflammatory cytokines upon stimulation with IL1β, IL18 and TLR agonists. Thus the immunological phenotype of IRAK4-/- mice is consistent with that of IRAK4-deficient patients.

**Dysregulated IRAK signaling in cancer**

The link between inflammation and cancer dates back to 1863, when Rudolf Virchow first observed leukocyte-infiltrates in tumor tissues (99). Today, inflammation is considered an enabling characteristic, and key factor, which can drive cancer pathogenesis (100-102). Moreover, it has become evident that an inflammatory microenvironment is an important general characteristic of human tumors (103). Not surprisingly, many environmental cancer risk factors are associated with chronic inflammation. For example, it is well established that the induction of inflammation by bacterial and viral infections increases cancer risk (104). Similarly, tobacco smoke and obesity are tumor-promoting factors through their ability to trigger chronic inflammatory signaling (105, 106).

IL1β, an important pro-inflammatory cytokine, and activator of IRAK signaling, plays a direct role in tumor cell growth (107), angiogenesis (108), invasion (109, 110), drug resistance (111), and metastasis (112). Similarly, depending on the tumor cell context, TLRs can
participate in a myriad of protumor responses (113). Thus, as necessary mediators of IL1R and TLR-inflammatory signaling, the IRAK family kinases represent rational cancer drug targets.

**Lymphoid Malignancies**

Cancer-specific dependencies on IRAK signaling were first uncovered through the discovery of oncogenically active MyD88 mutations in activated B cell-like diffuse large B cell lymphoma (ABC DLBCL) (114). Notably, in a large set of tumor biopsies, sequence analysis of the MyD88 coding region revealed that 29% of ABC DLBCL tumors harbored the L265P single amino acid substitution within the MyD88 TIR domain. This mutation was lacking in other DLBCL subtypes, including germinal center B-cell like (GCB)-DLBCL and Burkitt’s lymphoma. The L265P mutant promoted cell survival through spontaneous assembly of a protein-signaling complex containing IRAK1 and IRAK4, leading to IRAK4 kinase activation, IRAK1 phosphorylation, and activated JAK-STAT and NF-κB signaling. Strikingly, in ABC DLBCL cell lines harboring L265P MyD88, RNAi-mediated knockdown of MyD88, IRAK4 or IRAK1 eliminated NF-κB activation, and induced rapid apoptosis. Thus, in this context, sustained MyD88-IRAK signaling is necessary for ABC DLBCL pathogenesis and tumor cell survival. In shRNA rescue experiments, IRAK4 kinase activity was necessary to prevent RNAi-induced apoptosis; conversely kinase-dead IRAK1 was capable of rescuing RNAi-induced apoptosis. Thus in ABC DLBCL IRAK1 and IRAK4 have divergent kinase activities, and interestingly, IRAK1 appears to possess non-catalytic pro-survival activity. Ultimately this study supports the development of IRAK4 selective kinase inhibitors for the treatment of tumors harboring oncogenic MyD88 mutations.

In a related lymphocytic hematological malignancy, Waldenstrom’s Macroglobulinemia (WM), suppression of IRAK signaling appears to be a promising therapeutic approach. The common somatic L265P mutation of MyD88 is even more prevalent in WM, occurring in 91% of patients (115). Treon and colleagues first reported IRAK1/4 kinase inhibitor-mediated apoptosis
of primary MyD88 L265P-expressing cells derived from WM patient marrow (116). This study was the first to uncover Bruton’s tyrosine kinase (BTK) as an important binding partner of MyD88 L265P, and showed that the L265P mutant activates BTK in WM. Since, BTK and IRAK signaling similarly converge on NF-κB target gene transcription, the authors hypothesized that combined BTK and IRAK inhibition would provide a synergistic apoptotic effect. Indeed, potent synergistic WM cell killing was observed when combining the prototype BTK inhibitor, ibrutinib, with a small-molecule inhibitor of IRAK1/4. However, unlike in ABC DLBCL, the relative contribution from either IRAK1 or IRAK4 to WM cell survival is still unclear and remains an important question for future studies. Thus far, data collected from ongoing phase II trials with ibrutinib points towards very promising activity in WM (117). Combining ibrutinib with an IRAK kinase inhibitor would therefore be a rational approach and may provide a synergistic efficacy profile for WM patients.

**Myeloid Malignancies**

Recently, we have reported the activation and overexpression of IRAK1 in Myelodysplastic Syndrome (MDS) and Acute Myeloid Leukemia (AML) (118). IRAK1 is a validated target of miR-146a, a microRNA that drives the pathogenesis of MDS patients harboring a common cytogenetic abnormality, del(5q) (119-121). However, IRAK1 activation appears to be a more general feature of MDS and AML and is readily observed in non-del(5q) patients, suggesting alternate mechanisms of IRAK1 dysregulation. One possibility explaining IRAK1 activation in non-del(5q) MDS patients, is through the reported overexpression of TLR1/2/6 (122). In support of this thesis, marrow cells from MDS patients harboring mutations within TLR2 exhibit markedly increased pIRAK1 levels (122). Our research examined a small-molecule inhibitor of IRAK1/4 (IRAK-Inh) in MDS. The pharmacological effects of IRAK-Inh included a dose-dependent effect on cell growth, apoptosis, and progenitor cell function. Additional validation was carried out through RNAi-mediated knockdown of IRAK1 in MDS/AML cells, similarly resulting in apoptosis, and impaired MDS/AML-progenitor cell function.
Interestingly small-molecule inhibition of IRAK1/4 was ineffective against primary AML cells as well as the promyelocytic leukemia HL60 cell line. A potential explanation was offered through integrated gene-expression analysis, which uncovered compensatory upregulation of BCL2 mRNA in IRAK-Inh treated AML cells. A combined BCL2/IRAK inhibitory strategy was deployed to examine AML cell dependency on BCL2 activity within the context of inhibited IRAK1. The combination of the BH3 mimetic, ABT263, and IRAK-Inh elicited powerful collaborative cytotoxicity against all MDS and IRAK-Inh-refractory AML cells tested, both in primary cell culture and tumor xenograft settings. Furthermore, in vitro studies of this drug combination against normal CD34⁺ cord blood cells suggested a reasonable therapeutic window. It remains to be seen whether this drug combination will prove effective in other tumor types, but these findings implicate IRAK1 as a drugable target in MDS and AML.

A proximal IRAK signaling adaptor, IL1R-accessory protein (ILRAP), has similarly been implicated as a promising molecular target for MDS/AML (123). IL1RAP was found to be overexpressed on the surface of HSCs of AML and high-risk MDS patients and serves as an independent prognostic indicator in normal karyotype (NK)-AML. RNAi-mediated knockdown of ILRAP decreases clonogenicity and increases AML cell death. More recently, selective killing of AML HSCs, and Chronic Myeloid Leukemia (CML) cells, has been achieved through antibody-based targeting of IL1RAP (124).

Tumor-infiltrating immune cells, particularly monocytes, exhibit characteristic immune tolerance after first-exposure to cancer cells. This cancer-induced immune tolerance stems from the downregulation of pro-inflammatory genes. Interestingly, during this phenomenon monocytes isolated from CML patients exhibit upregulated IRAK3 expression (125). Thus IRAK3 becomes expressed in immune cells during cancer tolerance, acting to rapidly deactivate anti-tumor inflammatory responses through suppressing the activation of IRAK1/2/4 and thus dampening NF-κB activity. Increased monocytic IRAK3 expression is mediated by tumor-secreted hyaluronan (HA) through engagement of monocyte/macrophage-expressed CD44 and
TLR4. Since IRAK3 expression is limited to monocytes and macrophages, small-molecule activators of IRAK3 may prove useful in activating host anti-tumor responses. Indeed, IRAK3-deficient mice are resistant to tumor cell growth and provide experimental proof-of-concept for this approach (126).

**Solid Tumors**

Although there is less literature regarding the IRAK family kinases in the context of solid tumors, there is nascent evidence implicating IRAK1/4 signaling in melanoma (127). IRAK1/4 are overexpressed and activated in melanoma cell lines, and, as measured through IHC, pIRAK4 is highly expressed in primary melanoma biopsies. A small-molecule IRAK1/4 inhibitor was effective in sensitizing melanoma cell lines to chemotherapy, and combined vinblastine plus IRAK1/4 inhibition has shown significant survival benefit, as compared to monotherapy, in a melanoma xenograft model. Thus understanding the mechanism by which IRAK inhibition sensitizes melanoma cells to chemotherapies may lead to the discovery of more effective targeted melanoma therapies.

**Pre-clinical Discovery and Development of IRAK kinase inhibitors**

While at Amgen, Powers and colleagues first reported the discovery and structure-activity relationships (SAR) of small-molecule inhibitors of IRAK family kinases (128). High-throughput screening efforts resulted in the identification of a novel series of N-acyl-2-aminobenzimidazoles that had sub-micromolar IRAK4, and micromolar IRAK1 IC$_{50}$ values. Shortly following this, the same group reported crystal structures of IRAK4 kinase in complex with a similar N-acyl-2-aminobenzimidazole (129). The structures revealed a unique tyrosine gatekeeper residue, which creates an unusual ATP-binding site. Interestingly, when compared against the entire kinome, the tyrosine gatekeeper residue is exclusive IRAK family of kinases. Since the gatekeeper residue has been shown to play a critical role in kinase/small-molecule selectivity, the design of highly selective IRAK kinase inhibitors could be tractable through this
unique molecular feature. Unlike IRAK4, the crystal structure of IRAK1 remains to be solved. The IRAK1 crystal structure will likely prove useful in the synthesis of IRAK1- and IRAK4-selective kinase inhibitors.

A series of additional efforts undertaken at UCB Pharma uncovered increasingly potent compound classes, including imidazo[1,2-a]pyridino-pyridines and benzimidazolo-pyridines as low nM IC$_{50}$ IRAK4 and IRAK1 inhibitors (130-132). However, there remains a paucity of published literature surrounding the effects of these compounds against disease models, including cancer.

Nimbus Discovery is currently focused on the development of selective IRAK4 inhibitors. They have exploited high-energy hydration sites to design three selective nanomolar IRAK4 ligands (133). The K$_i$ of ND-346, ND-2110 and ND2158 for IRAK4 are 50, 7.5 and 1 nM, respectively (134). Each candidate small-molecule has demonstrated favorable pharmacokinetic/pharmacodynamic (PK/PD) characteristics and efficacy in several murine autoimmune disease models (134). The gain-of-function L265P mutation in MyD88 found in 29% of patients with ABC DLBCL served as rationale for Nimbus to pursue the combination of their selective IRAK4 inhibitor (ND-2158) with leading BTK, SYK and PI3Kδ inhibitors. Indeed, ND-2158 synergistically combines with either BTK, SYK, or PI3Kδ inhibitors to inhibit proliferation of the OCI-LY10 ABC DLBL cell line (135). Ultimately, these approaches will likely bring a selective IRAK4 inhibitor into clinical development for lymphoma, wherein the clinical development strategy will revolve around combination trials with active FDA-approved agents.

With the recent approval of the covalent BTK inhibitor, ibrutinib, there has been a renewed interest in the development of irreversible kinase inhibitors. These small-molecule inhibitors form a covalent bond with a nucleophilic cysteine residue within the kinase ATP-binding pocket, thus irreversibly inactivating the target kinase. An effort undertaken by Gray and colleagues, aimed at developing irreversible covalent inhibitors of JNK1/2/3, serendipitously
led to the discovery of a covalent kinase inhibitor of IRAK1 (136). In addition to JNK 1, 2, 3, KINOMEscan profiling revealed that the JNK-IN-7 phenylaminopyrimidine tool compound bound to directly to IRAK1, exhibiting an enzymatic IC$_{50}$ of ~10 nM. Sequence alignment and subsequent examination of the IRAK4 crystal structure revealed C276 as the IRAK1 candidate reactive cysteine residue. Biochemical profiling demonstrated that JNK-7-IN was also capable of inhibiting Pellino 1 E3 ligase activity, suggesting that IRAK1 is a bona fide intracellular target. Thus JNK-IN-7 may serve as a useful pharmacological probe to examine IRAK1-dependent cellular functions, and as a lead compound for the further development of increasingly potent covalent IRAK1 kinase inhibitors.

Outside the realm of rationally designed small-molecule inhibitors, there is perhaps only a single report of a natural product with activity against IRAK family kinases. Ginsenoside Rb1 and it’s metabolite, compound K, both derived from the root of Panax ginseng, a widely used herbal medicine, selectively inhibit IRAK1, but not IRAK2 or IRAK4 activation (137). Compound K has been extensively studied and has exhibited anti-inflammatory, anti-tumor and anti-diabetic effects, however prior to this study the molecular mechanisms of this metabolite remained undefined. Orally administered ginsenoside Rb1 and compound K were capable of improving disease symptoms through IRAK1 inhibition, which resulted in reducing the expression of TNF-α, IL-1β, IL6 and NO in a TNBS-induced colitic murine model. Both metabolites inhibited LPS-induced IRAK1 phosphorylation, IKKβ phosphorylation, NF-κB activation, and ERK and p38 activation. Biochemical profiling is necessary to determine whether either metabolite can directly inhibit the kinase activity of IRAK1. However, if direct enzymatic inhibition is confirmed, these natural metabolites may eventually provide a novel scaffold for the rational design of small-molecule IRAK1 kinase inhibitors.
### Figures

#### Table 1.1. FDA-approved kinase inhibitors indicated for cancer.

<table>
<thead>
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<th>Agent</th>
<th>Target(s)</th>
<th>FDA-approved indication(s)</th>
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<td>Afatinib (Gilotrif, Boehringer</td>
<td>EGFR, HER2</td>
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<td>Ingelheim)</td>
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<td>Exelixis)</td>
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<tr>
<td>Crizotinib (Xalkori, Pfizer)</td>
<td>ALK, MET</td>
<td>ALK+ non-small cell lung cancer</td>
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<td>Melanoma with BRAF V600E mutation</td>
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<td>Myers Squib)</td>
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<td>Non-small cell lung cancer</td>
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<td>Pharmacyclics and J&amp;J)</td>
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<td>ERBB1)</td>
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<td>Target(s)</td>
<td>Indication</td>
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<tr>
<td>Tremetinib (Mekinist, GSK)</td>
<td>MEK1/2</td>
<td>Melanoma</td>
</tr>
<tr>
<td>Vandetanib (Capresla, IRR Pharms)</td>
<td>EGFR1 (HER1/ERBB1), RET, VEGFR2</td>
<td>Medullary thyroid cancer</td>
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<tr>
<td>Vemurafenib (Zelboraf, Roche)</td>
<td>BRAF</td>
<td>Melanoma with BRAF V600E mutation</td>
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Figure 1.1. Domain architecture and signaling pathway of IRAK family kinases. (A) Domain architecture, size, localization and expression pattern of human IRAK kinases. (B) IRAK signaling pathway.
Chapter 2: Targeting IRAK1 as a therapeutic approach for Myelodysplastic Syndrome

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Published in \textit{Cancer Cell}. 2013 Jul 8;24(1):90-104. PMID: 23845443.
Summary

Myelodysplastic Syndromes (MDS) arise from a defective hematopoietic stem/progenitor cell. Consequently, there is an urgent need to develop targeted therapies capable of eliminating the MDS-initiating clones. We identified that IRAK1, an immune modulating kinase, is overexpressed and hyperactivated in MDS. MDS clones treated with a small-molecule IRAK1 inhibitor (IRAK1/4-Inh) exhibited impaired expansion and increased apoptosis, which coincided with TRAF6/NF-κB inhibition. Suppression of IRAK1, either by RNAi or with IRAK1/4-Inh, is detrimental to MDS cells while sparing normal CD34+ cells. Based on an integrative gene expression analysis, we combined IRAK1 and BCL2 inhibitors and found that co-treatment more effectively eliminated MDS clones. In summary, these findings implicate IRAK1 as a drugable target in MDS.

Significance

Myelodysplastic syndromes (MDS) are heterogeneous diseases with few treatment options. One of the key challenges facing MDS treatment is the lack of effective medicines capable of providing a durable response. Here, we identify that Interleukin Receptor Associated Kinase-1 (IRAK1) is overexpressed and activated in MDS. Genetic or pharmacological inhibition of IRAK1 induces apoptosis and cell cycle arrest of MDS cells, and prolongs survival outcome in a human MDS xenograft model. In an attempt to understand the mechanism of IRAK1-Inh function and potential resistance, we identified a collaborative cytotoxic effect of combined IRAK1 and BCL2 inhibition on MDS cells. Our findings suggest that targeting IRAK1 may be an effective therapeutic strategy in MDS.
Introduction

Myelodysplastic syndromes (MDS) are hematologic malignancies defined by blood cytopenias due to ineffective hematopoiesis, and a predisposition to acute myeloid leukemia (AML) (35, 138). Ensuing hematologic complications are often fatal if untreated. Approximately 30% of MDS patients also develop aggressive AML due to acquisition of additional mutations in the defective hematopoietic stem/progenitor cell (HSPC) (36). MDS is most prominent in individuals over 60 years of age, and as a result of longer life expectancies, the incidence of MDS has escalated in recent years (139). Current treatment options for MDS include allogeneic HSC transplantation, demethylating agents, and immunomodulatory therapies (140). At present, the only curative treatment for MDS is HSC transplantation, an option unavailable to many of the older patients. Overall the efficacy of these treatments is variable, and generally life expectancies are only slightly improved as compared to supportive care. Targeted therapies have been effective in other myeloid diseases (141), and may also improve the clinical outcome in MDS by suppressing the malignant clone. Recent sequencing and gene profiling efforts have revealed insight into the underlying molecular and cellular defects in MDS-initiating cells. Despite this progress, one of the key challenges still facing MDS treatment is that molecular-targeted therapies do not exist and AML-like therapies have been disappointing.

MDS are genetically defined by somatic mutations and chromosomal abnormalities not only affecting epigenetic plasticity, ribosome function, spliceosome machinery, or activation of oncogenes but also immune dysfunction. Human miR-146a resides on chromosome 5q33.3, and its deletion occurs in 80% of all del(5q) MDS and AML (142). Low expression of miR-146a, also occurs in >25% of all MDS and in >10% of AML patients (119, 143, 144), and is part of an MDS diagnostic miRNA signature (143). Knockout of miR-146a results in an early onset of myeloid expansion in the marrow, and progression to more aggressive diseases such as lymphomas, marrow failure, and myeloid leukemia (120, 121). TRAF6 and IRAK1 are two immune-related targets of miR-146a (119, 145, 146), and as expected, miR-146a knockout mice
have a dramatic increase in TRAF6 and IRAK1 protein within the hematopoietic compartment (120, 121). TRAF6, a lysine (K)-63 E3 ubiquitin ligase, and IRAK1, a serine/threonine kinase, are interacting proteins and mediators downstream of Toll-like (TLR) and Interleukin-1 (IL1R) receptors. Activation of TLR or IL1R recruits a series of adaptor proteins resulting in phosphorylation of IRAK1 on Thr209. Phosphorylated IRAK1 binds to and activates TRAF6 resulting in NF-kB activation. Increasing clinical and biological data indicate that innate immune signaling is an important determinant of MDS pathophysiology (147-150). The goal of this study was to identify a drugable molecular target within the innate immune pathway and determine whether pharmacologic inhibition of this pathway is effective at suppressing the MDS clone.

Results

**IRAK1 is overexpressed and activated in MDS.**

IRAK1 mRNA expression was evaluated in two gene expression studies comparing normal and MDS CD34+ marrow cells (150, 151). Both studies revealed that IRAK1 transcript is overexpressed by approximately 2-fold in ~10-30% of MDS patients (Figure 2.1A; p = 0.036 and p = 0.05, respectively). An independent group of MDS patients segregated according to high (top 50%) and low (bottom 50%) IRAK1 expression revealed that high IRAK1 expression correlates with reduced overall survival (p = 0.035; Figure 2.S1A,B). IRAK1 protein expression was similarly overexpressed in marrow cells from 5 low/intermediate-risk MDS patients, 3 AML patients, and in 6 MDS/AML cell lines (Figure 2.1B-E), suggesting that IRAK1 may be a relevant molecular target in MDS.

IRAK1 is activated in response to lipopolysaccharide (LPS) or interleukin-1b (IL-1b), and subsequently becomes phosphorylated (p) at threonine-209 (pT209) (Figure 1F-G) (55). To determine the activation status of IRAK1 in MDS, we measured pT209-IRAK1 by immunoblotting marrow cells from 5 MDS patients. As shown in Figure 1B, IRAK1 protein is not only overexpressed but also highly phosphorylated at T209. To confirm these observations, we
examined normal mononuclear cells (MNC), cord blood \text{CD}34^+ \text{ cells}, and a panel of 6 MDS/AML-derived human cell lines. In accordance with MDS patients, IRAK1 is overexpressed and hyperphosphorylated at T209 in all the MDS/AML cell lines examined, but not in normal MNC or CD34^+ cells (Figure 2.1C,E). In contrast, phosphorylated IRAK1 is observed to a lesser extent in primary AML despite having overexpression of IRAK1 protein (Figure 2.1C), suggesting that activated IRAK1 is more pronounced and specific in MDS. Phosphorylation at Serine 376 (S376), a residue not implicated in IRAK1 activation, was not phosphorylated in any of the cell lines (Figure 2.1D). These findings indicate that IRAK1 is overexpressed and activated in MDS patients.

The level of IRAK1 protein expression is significantly higher relative to IRAK1 mRNA expression in MDS/AML cells, suggesting that IRAK1 is overexpressed in part through a post-transcriptional effect (Figures 2.1A&B, and Figure 2.S1C). Since IRAK1 is a validated target of miR-146a, a miRNA deleted and implicated in the pathogenesis of MDS (119, 120, 146), we evaluated whether loss of miR-146a results in derepression of IRAK1 protein in MDS cells. We designed and overexpressed a retroviral miR-146a decoy in MDSL cells, which results in ~80% downregulation of endogenous miR-146a (Figure 2.S1D). Knockdown of miR-146a in MDSL cells resulted in ~3-fold increase in IRAK1 and TRAF6 protein (another validated miR-146a target). Furthermore, miR-146a expression inversely correlated with total IRAK1 mRNA/protein and phosphorylated IRAK1 in MDS/AML cell lines and MDS patient cells (Figure 2.S1E-G). Although multiple mechanisms may contribute to increase IRAK1 expression and/or activation, loss of miR-146a may represent a key event in MDS resulting in constitutively active IRAK1. Notwithstanding, overexpression and activation of IRAK1 is a common feature and represents a molecular target in MDS.
**IRAK-Inh blocks TRAF6 and NF-kB activation.**

A small-molecule inhibitor of IRAK1 (IRAK1/4-Inh or IRAK-Inh), which selectively inhibits its kinase activity in a low micromolar range (IC\textsubscript{50} = 0.75 mM), has been initially developed for autoimmune disease (Figure 2.2A) (128, 152). To determine whether IRAK-Inh can effectively inhibit active IRAK1 in MDS/AML cell lines and patient cells, we treated cells with an escalating dose of IRAK-Inh (0-10 mM) for 24 hrs. pT209 was reduced in a dose-dependent manner in MDS/AML cell lines (Figure 2B and Figure S2A). At 10 mM IRAK-Inh, phosphorylated IRAK1 was reduced by ~70% in cell lines and patient marrow cells (Figure 2.2B-D). Examination of kinases with shared homology that may be targeted by the IRAK-Inh at higher concentrations revealed that only IRAK1 is a target of IRAK-Inh in malignant myeloid cells (Figure 2.S2A). Lentiviral-mediated IRAK1 or TRAF6 overexpression resulted in increased pT209 (without exogenous stimulation), which is also inhibited by IRAK-Inh by ~50% (Figure 2.S2B,C). Upon phosphorylation, IRAK1 simultaneously undergoes TRAF6-mediated K63-linked ubiquitination (Figure 2A), which is another indication of its active state (72). In the presence of IRAK-Inh, immunoprecipitated (IP) IRAK1 exhibits reduced phosphorylation and K63-linked ubiquitination (Figure 2.2E).

TRAF6 forms a signaling complex with pIRAK1, resulting in IKK complex activation and subsequent NF-kB (RelA/p65) nuclear DNA-binding (Figure 2.2A). As expected, TRAF6 overexpression induces phosphorylation of IKKa/IKKb, the two catalytic proteins within the IKK complex (Figure 2.2F). In the presence of IRAK-Inh (10 µM for 24 hrs), vector- and TRAF6-expressing cells exhibit reduced pIKKa/IKKb (Figure 2.2F), but not relevant MAP kinases (p38 or ERK). Inhibition of p52 processing, which is a measure of non-canonical NF-kB activation and is independent of TRAF6 (Figure 2.2A), was also completely blocked by the IRAK-Inh (Figure 2.2A,F). In addition, DNA bound and active RelA/p65 was decreased by ~50% in MDS/AML cell lines by IRAK-Inh, indicating that IRAK-Inh effectively blocks IRAK1-mediated activation of NF-kB in MDS/AML cells (Figure 2.2G). Lastly, TRAF6 undergoes K63-
autoubiquitination, which is a necessary step prior to NF-kB activation (Figure 2.2A). Treatment with IRAK-Inh also coincides with reduced polyubiquitinated TRAF6 (Figure 2.2H). Taken together, IRAK-Inh effectively blocks IRAK1 function as is evident by reduced levels of phosphorylated and K63-ubiquitinated IRAK1, reduced autoubiquitination of TRAF6, and impaired NF-kB nuclear DNA binding (Figure 2.2A).

**Cytostatic effect of IRAK-Inh on MDS progenitor function and cell growth.**

MDS/AML cell lines with hyperphosphorylated IRAK1 (Figure 2.1D) were evaluated for sensitivity to IRAK-Inh. MDS/AML cell lines exposed to increasing concentration of IRAK-Inh were cultured for up to 6 days in vitro (Figure 2.3A). A significant, dose-dependent impairment of MDSL, TF1, and THP1 cell proliferation was observed in the presence of IRAK-Inh (Figure 2.3A). In contrast, the proliferation of normal cord blood-derived CD34+ cells, which do not exhibit activation of IRAK1, was not affected with even high doses of IRAK-Inh (Figure 2.3A). Despite having hyperphosphorylated IRAK1 (Figure 2.1C,D) and exhibiting reduced pIRAK1 after IRAK-Inh treatment (Figure 2.2D), the viability of HL60 cells and primary AML marrow cells was only modestly reduced with IRAK-Inh (Figure 2.3A, Figure 2.S3A,B). In support of this observation, the inhibitory effect of the IRAK-Inh on the cell lines correlated with the level of phosphorylated IRAK1 ($R^2 = 0.36$; Figure 2.3B). In parallel, cell viability was examined after 48 hrs of treatment with the IRAK-Inh. All cell lines exhibited a modest increase in apoptosis as measured by AnnexinV+ (Figure 2.3C). To investigate whether IRAK-Inh affects cell cycle progression, MDSL cells were treated with 10 mM IRAK-Inh for up to 6 days and evaluated by BrdU/7AAD staining (Figure 2.3D). Consistent with reduced proliferation (Figure 3A), MDSL cells treated with IRAK-Inh have altered cell cycle kinetics: there are fewer viable MDSL cells in S phase ($4.3\% \pm 0.4$ versus $14.2\% \pm 0.2$; $p = 0.0002$) and increased proportion in G0/G1 ($62.7\% \pm 1.1$ versus $54.5\% \pm 0.7$; $p = 0.008$) (Figure 2.3D). In addition, the proportion of sub-G0
cells was significantly increased in IRAK-Inh-treated cells (5.4% ± 0.3 versus 1.8% ± 0.2; p = 0.003).

The effect of IRAK-Inh on leukemic progenitor function was evaluated in methylcellulose containing the IRAK-Inh. Normal CD34+ formed equivalent number of colonies with moderate skewing of erythroid and granulocytic/macrophagic progenitors (p < 0.05) when treated with IRAK-Inh (Figure 2.S3C). In stark contrast, all MDS/AML cells formed significantly fewer colonies when treated with IRAK-Inh (Figure 3E). As an alternative approach, MDS/AML cells were pre-treated with IRAK-Inh for 24 hours and then plated in methylcellulose (Figure 2.S3E). After transient exposure (24 hr) to IRAK-Inh (10 µM), MDS/AML cells, but not normal CD34+, formed significantly fewer colonies, suggesting that IRAK-Inh suppresses the function of the disease-propagating cell (Figure 2.S3F,G). Increased apoptosis, impaired cell cycle progression, and impaired progenitor function after IRAK-Inh treatment is not a consequence of induced differentiation, as IRAK-Inh-treated MDS/AML cells did not undergo noticeable myeloid differentiation in vitro (Figure 2.S3H). To determine whether the effects of IRAK-Inh can be rescued by activating the innate immune pathway downstream of IRAK1 (Figure 2.2A), we overexpressed TRAF6 and then measured the cellular toxicity of the IRAK-Inh. Although IRAK-Inh partially suppress NF-kB in TRAF6-overexpressing cells (Figure 2.2E), forced expression of TRAF6 in MDSL cells can overcome the inhibitory effects of IRAK-Inh and restore cell viability and progenitor function in IRAK Inh-treated cells (Figure 2.S3H,I). Collectively, these results suggest that IRAK-Inh is effective in selectively inhibiting the viability and function of MDS progenitor cells while sparing normal CD34+ cells by directly targeting the innate immune pathway.

We evaluated marrow cells from 7 MDS and 3 AML patient samples with various cytogenetic features to determine whether IRAK1 inhibition is also effective in primary patient cells (Table S1). MDS marrow cells treated with IRAK-Inh for 48 hrs failed to expand (Figure 2.3F) and exhibited increased levels of apoptotic cells, except for MDS-07 (Figure 2.3G).
In contrast, vehicle control-treated MDS cells expanded nearly 2-fold during this time and had significantly fewer apoptotic cells (Figure 2.3F,G). AML marrow cells treated with IRAK-Inh continued to grow similar to control-treated cells (Figure 2.S3B). Since MDS and AML stem/progenitor cells are clonal and the disease-propagating cells form colonies in methylcellulose, we also evaluated the effects of the IRAK-Inh on colony formation. Whether continuously treated (Figure 2.3H) or briefly exposed to IRAK-Inh, (Figure 2.S3G), MDS marrow cells formed significantly fewer colonies in the presence of IRAK-Inh. In contrast, IRAK-Inh did not affect AML or control CD34+ cell progenitor function (Figure 2.2H, Figure 2.S3C,D). These findings indicate that IRAK-Inh selectively inhibits growth and progenitor function of primary MDS marrow cells, and that IRAK-Inh sensitivity is a general feature of MDS, independent of existing genetic features.

IRAK-Inh ameliorates disease in a human xenograft model using an MDS-derived cell line.

Primary MDS patient samples remain difficult to engraft into immunodeficient mice, typically with less than 5% marrow engraftment and no evidence of disease (153, 154). To circumvent this limitation, we developed a xenograft model using an MDS patient-derived cell line (MDSL), which has retained phenotypic and cellular characteristics of MDS (155, 156). Consistent with non-transforming MDS subtypes, the MDSL cell line does not form overt leukemia in NSGS or NSG mice. Instead, MDSL cells engraft into the marrow and gradually expand over time within the marrow, spleen, and peripheral blood as a non-blast/incompletely differentiating myeloid population (Figure 2.S4A). Xenografted mice develop progressive anemia, thrombocytopenia, and extramedullary hematopoiesis and eventually succumb to disease (Figure 2.S4B). Disease progression and MDSL expansion in the marrow is accompanied by depletion of normal mouse hematopoietic cells and a hypocellular marrow (Figure 2.S4C,D).
To determine whether IRAK-Inh can delay human MDS-like disease in vivo, MDSL were pre-treated with IRAK-Inh (10 µM) for 24 hrs in vitro and subsequently injected intravenously (i.v.) into NSG (5×10^6 cells) and NSGS (1×10^6 cells) recipient mice (Figure 2.4A). This approach permitted us to evaluate the cell autonomous effect of IRAK-Inh on MDS cell viability and engraftment without altering endogenous IRAK1 function in the mouse. As shown in Figure 2.4B, pretreatment with IRAK-Inh significantly delayed the MDS-like disease in NSG mice (median survival = 80 days vs 68 days; p = 0.0065) and in NSGS mice (median survival = 38 days vs 29 days; p = 0.028). NSGS mice transplanted with IRAK-Inh-treated MDSL cells also had significantly improved red blood cell numbers (p = 0.0085), hemoglobin (p = 0.012), hematocrit (p = 0.015) and platelets (p = 0.02) (Figure 2.4C). In addition, the human MDSL graft was reduced from ~10% to 3% (Figure 2.4D). Morphological assessment confirmed normal RBC and reduced MDS grafts in the peripheral blood and marrow of mice transplanted with IRAK-Inh-treated MDSL cells (Figure 2.4E). To demonstrate that the IRAK-Inh can also ameliorate disease after cells have engrafted, MDSL were injected i.v. into NSG mice, followed by intraperitoneal (i.p.) injection of IRAK-Inh (3X/week; 2.12 mg/kg) (Figure 2.4A). Mice receiving IRAK-Inh maintained HCT and Hb levels while control mice exhibited a progressive anemia (Figure 2.4F). Within ~7 days of receiving IRAK-Inh, mice had reduced human graft in the peripheral blood (Figure 2.4G,H). These findings indicate that IRAK-Inh targets the disease-propagating cell and provides a significant survival benefit in a xenograft model of human MDS.

**Knockdown of IRAK1 protein induces apoptosis and impaired clonal-progenitor function.**

To validate the effects and specificity of the IRAK-Inh, lentiviral vectors encoding independent short hairpin RNAs (shRNA) targeting IRAK1 were transduced into MDS/AML cell lines and MDS patient marrow cells. We confirmed targeting of IRAK1 by immunoblotting and quantitative RT-PCR (qRT-PCR) (Figure 2.5A, Figure 2.S5). Although IRAK-Inh induced only a modest apoptotic affect (Figure 2.3B), all MDS/AML cell lines with depletion of IRAK1 correlated
with a significant increase in apoptosis (AnnexinV+ cells; Figure 2.5B). Knockdown of IRAK1 in CD34+ cells did not induce apoptosis, indicating that IRAK1 is dispensable for normal CD34+ viability (Figure 2.5B). MDS/AML cell lines and MDS patient samples with knockdown of IRAK1 also exhibited a significant decrease in progenitor colonies in methylcellulose (Figure 2.5C,D). Normal CD34+ were not sensitive to loss of IRAK1 and formed colonies at the same level to control-transduced CD34+ cells (Figure 2.5C).

To examine the effects of IRAK1 depletion in vivo, MDSL cells were transduced with a doxycycline (DOX)-inducible shIRAK1 (pTRIPZ; Figure 2.5D). Increasing amounts of DOX in vitro demonstrated a dose-dependent deletion of IRAK1 mRNA and protein, as well as reduced cell viability (Figure 2.5E, Figure 2.5F). The effects of IRAK1 knockdown were rescued by expressing a hairpin-resistant IRAK1 cDNA (Figure 2.5G). Transduced MDSL cells were injected into NSG mice, and six days post-transplant, mice were given chow with or without DOX. Expression of the shRNA is observed in the marrow, blood, and spleen of DOX-treated mice, but not expressed in control mice (Figure 2.5I). Without IRAK1 knockdown (minus DOX or non-transduced parental MDSL cells), mice developed the MDS-like disease (Figure 2.5F-I, Figure 2.5J). In contrast, IRAK1 knockdown (plus DOX) reduced engraftment of MDS cells (PB: ~15% versus 5% [Figure 2.5F]; BM: ~40% vs 10% [Figure 2.5G]) and spleen size (Figure 2.5H), and significantly delayed mortality in mice (p = 0.001; Figure 2.5I). Collectively these data suggest that genetic depletion of IRAK1 results in reduced viability and growth of MDS/AML progenitor cells in vitro and in vivo. In addition, a more profound effect on MDS/AML survival and progenitor function was observed following depletion of IRAK1 as compared to the IRAK-Inh.
Expression profiling following deletion or inhibition of IRAK1 reveals overlapping gene signatures and a compensatory increase in BCL2

We performed a gene expression analysis to (1) gain insight into the molecular consequences of inhibiting IRAK1 in MDS cells, and (2) define an underlying mechanism for the discrepancy between IRAK-Inh and shIRAK1 apoptotic threshold in MDS/AML cells. MDSL cells were either transduced with shIRAK1 (or control) or treated with IRAK-Inh (or DMSO) for 48 hours, followed by RNA collection for a microarray analysis (Figure 2.6A). At this time point, we observe >50% knockdown of IRAK1 mRNA by the shRNA and minimal effect on cell viability. Inhibition of IRAK1 by either approach in MDSL resulted in ~180 differentially expressed genes (Figure 2.6A). We searched for previously defined expression signatures that overlap genes regulated by both IRAK-Inh and shIRAK1 by using gene set enrichment analysis (GSEA) (157) (Figure 2.6A). Of the top 10 significant GSEA sets in each group, 3 gene sets were identical between IRAK-Inh and shIRAK1 MDSL cells (Figure 2.6B), indicating that IRAK1 is effectively targeted by both approaches and that inhibition by either approach induced a similar transcriptional response. Of the top GSEA sets, survival (IL6_Starve_Up) and cell cycle/proliferation (Cell_Cycle, and MM_CD138_PR_vs_REST) were the most significant in both IRAK-Inh and shIRAK1 experimental groups (Figure 2.6B). To better understand the cellular and molecular consequences following IRAK1 inhibition/deletion, we examined the Gene Ontology categories using ToppGene (158). Knockdown of IRAK1 resulted in down regulation of genes involved in chromatic assembly, DNA binding, and RNA metabolism (Figure 2.6C). IRAK-Inh treatment resulted in downregulation of genes involved in cell migration and adhesion, inflammatory response, and cytokine-mediated signaling (Figure 2.6C).

Despite the overlap in gene sets, we examined genes that could explain the discrepancy between IRAK-Inh and shIRAK1 in inducing apoptosis of MDS/AML cells. Although IRAK-Inh upregulated pro-apoptotic genes (e.g., BIM, CASP10, RIPK1), downregulation of anti-apoptotic Bcl2-family genes was not evident. As an example, BCL2 mRNA and protein was not
downregulated in IRAK-Inh-treated cells; surprisingly, BCL2 expression was increased in several of the cell lines examined, an effect not observed in shIRAK1-expressing cells (Figure 2.6D-F). This observation prompted us to speculate that a subset of MDS/AML progenitors escape IRAK-Inh induced apoptosis because of a compensatory upregulation or inefficient downregulation of BCL2.

**Combined inhibition of IRAK1 and BCL2 cooperates to target MDS clonal-progenitor function.**

We examined the survival dependence on BCL2 function in IRAK-Inh-treated cells by utilizing a BH3-mimetic (ABT-263, Abbott Laboratories). Administration of IRAK-Inh (10 µM) or ABT-263 (0.1 µM) alone had modest effects in inhibiting MDS/AML cell line growth and survival (Figure 2.7A,B). Strikingly, co-treatment of the MDS/AML cells with IRAK-Inh and ABT-263 significantly and synergistically inhibited cell growth and survival (Figure 2.7A,B). In particular, HL60 cells, which were refractory to the inhibitory effects of IRAK-Inh or ABT-263 alone, are extremely sensitive to the combined treatment with ABT-263 (Figure 2.7A,B and Figure 2.S6). In addition, MDS/AML cell lines and MDS patient progenitor colonies were significantly impaired with the combination treatment of IRAK-Inh and ABT-263 (Figure 2.7C,D). For AML patient cells that are not sensitive to IRAK-Inh treatment alone (Figure 2.S3D), co-treatment with ABT-263 also resulted in reduced leukemic progenitor function (Figure 2.7E,F). The viability and hematopoietic progenitor function in methylcellulose of normal CD34+ cells was not affected by the individual or combination treatment of IRAK-Inh and ABT-263 (Figure 2.S3C).

Moreover, we investigated whether combined IRAK1 and BCL2 inhibition could delay human MDS-like disease in vivo. MDSL cells were treated with IRAK-Inh (10 µM) and/or ABT-263 (0.1 µM) for 48 hrs in vitro and subsequently injected i.v. into NSGS mice (Figure 2.4A). Treatment with IRAK-Inh or ABT-263 alone significantly delayed the MDS-like disease in NSG
mice with a median survival of ~35 days (versus 28 days with DMSO treatment) (Figure 2.7G).

Mice receiving MDSL co-treated with IRAK-Inh and ABT-263 exhibited a significantly enhanced survival (43 days) as compared to individual drugs or DMSO (Figure 2.7G). Not only was survival extended, but also mice transplanted with cells pre-treated with the drug combination had improved red blood cell, hemoglobin, and platelet counts (not shown). In conclusion, inhibition of IRAK1 function with a small-molecular inhibitor may represent a treatment to inhibit MDS clone function and viability, while co-treatment with ABT-263 results in enhanced cytotoxicity.

Discussion

MDS is fatal in majority of patients as a result of marrow failure, immune dysfunction, and/or transformation to overt leukemia. Although allogeneic HSC transplantation can be curative, many MDS patients do not qualify for this treatment. Instead, these patients receive supportive care and transfusions to ameliorate their disease complications. Unfortunately, the MDS clones persist in the marrow and the disease invariably advances (159). For advanced disease or high-risk MDS, patients may also receive immunosuppressive therapy, epigenetic modifying drugs, and/or chemotherapy (160). Despite recent progress, most MDS patients exhibit treatment-related toxicities or relapse (161). Identification of molecular targets is essential to improve outcome and eliminate the MDS-causing clones. Herein, we identified and characterized IRAK1 as a therapeutic target for MDS.

IRAK1 is a serine/threonine kinase that mediates signals from TLR/IL1R to NF-kB under normal conditions (162). Following receptor activation, MyD88 recruits IRAK4 and IRAK1, resulting in IRAK1 hyperphosphorylation. IRAK1 phosphorylation at Thr-209, which is mediated by upstream signals or through autophosphorylation, is a key post-translational modification and a hallmark of its activation (55). Once phosphorylated, IRAK1 binds TRAF6 and undergoes K63-linked ubiquitination (72). This interaction between IRAK1 and TRAF6 initiates a signaling
cascade resulting in NF-κB nuclear translocation (72). Small-molecule inhibitors targeting IRAK1 have been originally developed for autoimmune and inflammatory diseases (152, 163). Given that the TRAF6/IRAK1 signaling complex remains in an activated state in MDS (119, 164, 165), it is not surprising that inhibiting this complex may have a therapeutic benefit in MDS, and represent a viable approach to inhibit NF-kB preferentially in malignant clones with activated IRAK1. Notably, most NF-kB inhibitors to date have been disappointing for the treatment of myeloid malignancies due to toxicity (166).

Although IRAK1 mRNA is overexpressed in a subset of MDS patients, the level of expression rarely exceeds 2-fold. However, deletion and reduced expression of miR-146a is a common event in MDS as it resides within the deleted region on chr 5q and its expression is reduced in a large subset of normal karyotype MDS (119, 144). TRAF6 and IRAK1 are two targets of miR-146a, and germline knockout of miR-146a results in derepression of TRAF6 and IRAK1 protein (120, 121). We confirmed a similar effect in MDS cells (Figure 2.S1D). This suggests that IRAK1 is transcriptionally and translationally upregulated in MDS patients making it a relevant molecular target. IRAK1 mRNA/protein is also overexpressed in subsets of AML patients (Figure 2.S2) (167), supporting our observations that targeting IRAK1 may extend to high-risk MDS and AML with active IRAK1.

Phosphorylation and activation of IRAK1 can also occur by gain-of-function mutations or aberrant expression of upstream signaling molecules. For example, human lymphomas with oncogenically active MyD88 mutations have constitutive IRAK1 phosphorylation and NF-kB activation, and are sensitive to IRAK inhibitors (168). In Fanconi anemia, IRAK1 exists in a hyperphosphorylated (higher-molecular weight) state, potentially as a consequence of aberrant TLR8 signaling (169). Of note, mutations in MyD88 or TLR8 have not been reported in MDS or AML, suggesting that alternate molecular alterations activate IRAK1 in MDS/AML. However, mutations of TLR2 are reported in ~10% of MDS patients (170), and a recent finding identified overexpression of interleukin-1 receptor accessory protein (IL1RAP) in HSC from MDS and
AML patients (171). Consistent with the hypothesis that hyperphosphorylation of IRAK1 in MDS may be due to aberrant activation downstream of the TLR/IL1R, a retrospective analysis revealed that chronic immune stimulation acts as a trigger and increases the risk for MDS and AML development (172). Collectively, these observations suggest that, in addition to downregulation of miR-146a, multiple other molecular alterations can converge on and activate IRAK1 in MDS.

According to our integrative gene expression analysis, inhibition of IRAK1 revealed that IRAK1 regulates genes involved in survival, cell cycle/proliferation, chromatic assembly/DNA binding, RNA metabolism, cell migration/adhesion, and inflammation (Figure 2.6C). These gene signatures are consistent with our observation that inhibiting IRAK1 in primary MDS marrow or cell lines results in delayed proliferation, reduced survival, and impaired progenitor function. IRAK-Inh was inefficient at downregulating pro-survival BCL2 genes, and in some cell lines, resulted in a compensatory increase in BCL2 expression, which is a common cellular response to cytostatic and cytotoxic therapies (173). To overcome this compensatory effect, we combined a BCL2 inhibitor (ABT-263) with IRAK-Inh, which resulted in potent collaborative cytotoxic effects in MDS and AML cells by inducing rapid and profound apoptosis (Figure 2.6S6). Notably, this effect was observed even in MDS/AML cells that did not exhibit a compensatory increase in BCL2 expression in response to IRAK-Inh. That IRAK-Inh is effective at suppressing MDS cells, but not AML, can be explained by: (1) an increased apoptotic threshold in high-risk MDS and AML due to higher expression of prosurvival BCL2 family members, thus necessitating co-treatment with a BCL2 inhibitor; (2) the level of activated IRAK1 in AML is lower as compared to MDS, suggesting differences in the molecular circuitry of IRAK1 activation; and/or (3) only select subtypes of AML are sensitive to IRAK-Inh while a larger proportion of MDS are sensitive.

The complexity and heterogeneity of MDS, and the lack of human xenograft models remain as obstacles to identifying and evaluating novel molecular targets for this disease. In addition, primary MDS cells do not efficiently engraft into immunodeficient mice (153, 154). To
overcome this limitation, we generated a human model using an MDS-derived patient cell line (MDSL). Consistent with phenotypic and cellular characteristics of MDS (155, 156), MDSL engraftment into NSG or NSGS immunodeficient mice results in a fatal and progressive anemia, thrombocytopenia, hypocellular marrow, and extramedullary hematopoiesis. Treatment of MDSL cells with IRAK-Inh or in vivo delivery of the inhibitor reduced the number of MDSL cells and delayed disease. This xenograft model represents an alternative to examine the mechanisms of low-risk MDS disease and a tool for preclinical studies using an MDS-derived patient cell line.

In summary, this report implicates IRAK1 as a drugable target for MDS. Inhibition with IRAK-Inh induces combined apoptosis and a cell cycle block, while inhibition with ABT-263 results in collaborative cytotoxicity in MDS cells. Future studies examining improved inhibitors of IRAK1 and additional in vivo analysis will be essential for determining the therapeutic applicability of IRAK1 inhibitors, as will be identifying the ideal target patient population based on active versions of IRAK1. Inhibitors targeting IRAK1 reveal an avenue for suppressing altered TLR/IL1R/TRAF6/NF-κB pathway and eliminating the MDS clone.

Experimental Procedures

Detailed experimental procedures are presented in the Supplemental Experimental Procedures.

Reagents and cell lines

The IRAK1 inhibitor (IRAK1/4 inhibitor or IRAK-Inh; Amgen Inc.) was purchased from Sigma-Aldrich (I5409). See Supplemental Information for additional information.

Primary samples

This study was approved by the Cleveland Clinic (Cleveland, OH) and at Fondazione IRCCS Ca’ Granda Ospedale Maggiore Policlinico, University of Milan (Milan, Italy). Informed consent was obtained according to protocols approved by the review boards of participating institutions.
The study conformed to the ethical standards set out in the Declaration of Helsinki. Diagnoses were reviewed at each of the participating centers and adapted, when required, to WHO 2008 criteria. For qRT-PCR analysis, presentation bone marrow aspirates were collected from 20 patients (CC1-CC20; Table S1). For functional studies, MDS mononuclear cells from bone marrow aspirates were obtained at diagnosis as part of a multicenter phase 2 trial based in Italy (MDS01-MDS08; Table S1). AML patient samples were acquired following informed consent and under the direction of IRB approved protocols. Human CD34+ umbilical cord blood (UCB) and adult marrow-derived mononuclear cells were obtained from Cincinnati Children’s Hospital.

**Survival analysis and growth curves**

AnnexinV analysis was carried out as previously described (165). Trypan blue exclusion was measured using an automated cell counter (BioRad TC10). For experiments beyond 48 hrs, cells were replenished with fresh media and drug every second day. For primary patient marrow cells, cell were treated with a single inhibitor dose and counted 24 and 48 hours later.

**Immunoblotting, DNA-binding assays, and qRT-PCR**

See Supplemental Information.

**shRNA-mediated knockdown of IRAK1**

The pLKO.1 constructs were obtained from the Lentiviral core at CCHMC. Puromycin resistance gene was replaced by green fluorescent protein (GFP). Two independent and validated pLKO.1-shIRAK1 constructs were obtained: TRCN0000000543 and TRCN0000000544. After validation, majority of experiments were performed with clone TRCN0000000544. For inducible knockdown, we used the TRIPZ doxycycline (DOX)-inducible shRNA system (OpenBiosystems) expressing shIRAK1 clone V2THS-132369. See Supplemental Information for additional details.
**Xenograftment of NOD/SCID-IL2Rg mice (NSG) and NSG-hSCF/hGM-CSF/hIL3 (NSGS)**

All animal experiments were performed in adherence to protocols approved by the Institutional Animal Care and Use Committee of Cincinnati Children’s Hospital Medical Center. MDSL cells were injected into the tail veins of 8-week old sublethally irradiated (250 Gy) NSG or NSG animals engineered to express human SCF, GM-CSF, and IL3 (NSGS) as previously described (174). In vivo delivery of IRAK-Inh approach is adapted from previous reports (175): IRAK-Inh was diluted in DMSO (5 mM) and further dissolved in sterile phosphate buffered saline (PBS; pH 7.2). Animals were injected i.p. with 2.12 mg/kg IRAK-Inh 3x weekly.

**Statistical Analysis**

Results are depicted as the mean ± standard error of the mean. Statistical analyses were performed using Student’s *t*-test. GraphPad Prism (v5, GraphPad) was used for statistical analysis.

**Accession Numbers**

The gene expression data from IRAK-Inh and IRAK1 RNAi can be found at the GEO database ([http://www.ncbi.nlm.nih.gov/geo](http://www.ncbi.nlm.nih.gov/geo)) using the accession number GSE46346.

**Acknowledgements**

This work was supported by Cincinnati Children’s Hospital Research Foundation, American Society of Hematology, National Institute of Health (RO1HL111103), and Department of Defense grants to D.T.S. Umbilical cord blood samples were received through the Normal Donor Repository in the Translational Core Laboratory at Cincinnati Children's Hospital, which is supported through the NIDDK Center's of Excellence in Experimental Hematology (P30DK090971). We thank Mt. Auburn and Christ Hospital (Cincinnati, OH) for collecting cord blood (CD34+) samples. We thank Drs. Ashish Kumar (Cincinnati Children's Hospital), Martin Carroll (University of Pennsylvania), and Yogen Saunthararajah (Cleveland Clinic) for AML
samples. We thank Jeff Bailey and Victoria Summey for assistance with mice ( Comprehensive Mouse and Cancer Core), and Nicholas Brown for technical assistance. MDSL cells were kindly provided by Dr. Kaoru Tohyama. The authors declare no competing financial interests.
Figure 2.1. IRAK1 is overexpressed and overactivated in MDS. (A) IRAK1 expression is obtained from two gene expression studies on CD34⁺ cells isolated from control and MDS marrows: Pellagatti (p = 0.036), Hoffmann (p = 0.05). (B) Mononuclear marrow cells from 3 control (MNC) and 5 MDS patients were examined for total and phosphorylated (p)-T209 IRAK1 by immunoblotting. (C) MNCs from two controls, three AML patients, and one MDS patient were examined for total and pIRAK1 T209 IRAK1 by immunoblotting. (D) MNCs and the indicated MDS/AML cell lines were examined for total, pIRAK1 T209, and pIRAK1 S376 by immunoblotting. pIRAK1 and IRAK1 were run on parallel gels (left panel). Densitometric values for pIRAK1 protein relative to GAPDH are summarized in the right panel. (E) Normal CD34⁺ cells, MDSL, and MDS patient marrow cells (MDS-02; same as in B) were examined for total and pIRAK1 T209 IRAK1 by immunoblotting. (F and G) THP1 cells were treated with 1 µg/ml LPS (F) or 10 ng/ml IL-1β (G) for the indicated time points and analyzed by immunobloting for total and pIRAK1 T209 (left panels). Densitometric values for pIRAK1 protein relative to IRAK1 are summarized in the right panel. Error bars represent ± SD. See also Figure S1 and Table S1.
IRAK1 by immunoblotting. (C) MNC from 2 controls, 3 AML patients, and 1 MDS patient were examined for total and pT209 IRAK1 by immunoblotting. (D) MNC and the indicated MDS/AML cell lines were examined for total, pT209, and pS376 IRAK1 by immunoblotting. pIRAK1 and IRAK1 were run on parallel gels (left panel). Densitometric values for pIRAK1 protein relative to GAPDH are summarized in the right panel. (E) Normal CD34+ cells, MDSL, and MDS patient marrow cells (MDS-02; same as in [B]) were examined for total and pT209 IRAK1 by immunoblotting. (F-G) THP1 cells were treated with 1 mg/ml LPS (F) or 10 ng/ml IL-1b (G) for the indicated time points and analyzed by immunobloting for total and pIRAK1 (pT209) (left panels). Densitometric values for pIRAK1 protein relative to IRAK1 are summarized in the right panel. Error bars represent +/- SD. See also Figure S1 and Table S1.
Figure 2.2. IRAK-Inh suppresses TRAF6 and NF-kB activation in MDS/AML. (A) Schematic of IRAK1/TRAF6/NF-kB signaling. IRAK-Inh (red line) inhibits IRAK1 kinase function and prevents TRAF6-mediated canonical NF-kB activation. (B) Protein lysates from THP1 cells and (C-D) MDS and AML marrow cells treated with IRAK-Inh for 24 hrs were evaluated by immunoblotting for total and pT209 IRAK1. Densitometric values for pIRAK1 protein relative to GAPDH or IRAK1 are shown below. (E) THP1 cells were treated with 10 µM IRAK-Inh for 24 hrs were isolated for immunoprecipitation (IP) of IRAK1. Immunoblot for ubiquitin (Ub) and pT209 IRAK1 (pIRAK1) was performed on the IP lysate. Shown is a representative image from 3 independent replicates. Densitometric values for Ub-IRAK1 protein relative to GAPDH are shown below. (F) Empty vector- and TRAF6-transduced THP1 cells were treated with IRAK-Inh (10 µM) for 24 hrs and examined by immunoblotting. (G) Nuclear lysates from THP1 and HL60
cell-lines treated with IRAK-Inh (10 µM) for 24 hrs were evaluated for p65 DNA binding activity. Shown is the mean of 3 independent replicates. Error bars represent +/- SEM. (H) THP1 cells were treated with 10 µM IRAK-Inh for 24 hrs were isolated for IP of TRAF6. Immunoblot for ubiquitin (Ub) and TRAF6 was performed on the IP lysate. Shown is a representative image from 3 independent replicates. Densitometric values for Ub-TRAF6 protein relative to GAPDH are shown below. See also Figure S2.
Figure 2.3. IRAK-Inh impairs MDS cell viability and progenitor function. (A) Viable cell growth of the indicated cell lines and normal CD34+ cells was assayed by trypan blue exclusion in the presence of IRAK-Inh (0-10 μM) for up to 6 days. (B) Relationship between IRAK-Inh sensitivity (IC50) and pIRAK1 levels (densitometric values of immunoblotted pIRAK1) was calculated for the indicated cell lines.
sensitivity (IC₅₀) and pIRAK1 levels (densitometric values of immunoblotted pIRAK1) was calculated for the indicated cell lines. (C) AnnexinV staining of the indicated cells was determined after 48 hr treatment with IRAK-Inh (10 µM). (D) Cell cycle analysis of MDSL cells treated with 10 µM IRAK-Inh for 6 days was determined by BrdU/7AAD incorporation. (E) Colony formation in methylcellulose was determined for the indicated cell lines treated with IRAK-Inh. Total colonies were scored after 10 days. (F-G) Marrow cells from MDS patients were evaluated for growth (trypan blue exclusion, F) and for apoptosis (AnnexinV staining, G) in the presence of IRAK-Inh. (H) Marrow cells from MDS patients were evaluated for colony formation in methylcellulose containing IRAK-Inh. *, p < 0.05; **, p < 0.01; ***, p < 0.001. Error bars represent +/- SD. See also Figure S3.
Figure 2.4. IRAK-Inh suppresses MDS xenografts. (A) Two experiment schemes were used to test the efficacy of IRAK-Inh in an MDS xenograft model: (left arm) MDSL cells were pretreated for 24 hr followed by transplantation into either NSG or NSGS mice; or (right arm) NSG mice were transplanted with MDSL cells and at day 6 mice were given IRAK-Inh (2.12 mg/kg i.p. 3x/week).

(B) Overall survival was determined for NSG (n = 4–5/group) and NSGS (n = 3–5/group) mice transplanted with MDSL cells treated with IRAK-Inh.

(C) Complete blood counts were performed at 68 (NSG) and 28 (NSGS) weeks postxenograftment of NSG and NSGS mice. Red blood cells (RBC), hemoglobin (Hb), hematocrit (HCT), and platelets (PLT) are shown for mice receiving control- or IRAK-Inh-treated MDSL cells.

(D) Flow cytometric analysis examining peripheral human graft in representative DMSO and IRAK-Inh NSG mice 28 days posttransplant.

(E) Wright-Giemsa marrow cytospins and blood smears from represented mice transplanted with control or IRAK-Inh-treated MDSL cells. Smears on all mice were performed at time of death of the control group. MDSL cells are indicated by arrowheads. Blood scale bar, 30 μm; BM scale bar, 5 μm.

(legend continued on next page)
mice were transplanted with MDSL cells and at day 6 mice were given IRAK-Inh (2.12 mg/kg i.p. 3X/week). (B) Overall survival was determined for NSG (n = 4-5/group) and NSGS (n = 3-5/group) mice transplanted with MDSL cells treated with IRAK-Inh. (C) Complete blood counts were performed at 68 (NSG) and 28 (NSGS) weeks post xenograftment of NSG and NSGS mice. Shown are red blood cells (RBC), hemoglobin (Hb), hematocrit (HCT), and platelets (PLT) for mice receiving control- or IRAK-Inh-treated MDSL cells. (D) Flow cytometric analysis examining peripheral human graft in representative DMSO and IRAK-Inh NSG mice 28 days post-transplant. (E) Wright-Giemsa marrow cytospins and blood smears from represented mice transplanted with control or IRAK-Inh-treated MDSL cells. Smears on all mice were performed at time of death of the control group. MDSL cells are indicated by arrowheads. Blood scale bar, 30 μm; BM scale bar, 5 μm. (F) NSG animals were transplanted with 1x10^6 cells/mouse. Six days post transplant, mice were injected with IRAK-Inh (2.12 mg/kg, 3X/week). Hematocrit (HCT) and hemoglobin (Hg) counts were performed on the indicated days (>4 mice per group). Error bars represent +/- SD. (G) Human MDSL engraftment was determined by flow cytometry by measuring hCD45 in peripheral blood. (H) Summary of MDSL engraftment in mice receiving IRAK-Inh (n = 5 per group). *, p < 0.05; **, p < 0.01. See also Figure S4.
Figure 2.5. IRAK1 is essential for MDS progenitor cell survival. (A) IRAK1 knockdown was confirmed by qRT-PCR in cells expressing a control or shIRAK1-expressing lentiviral vector. (B) AnnexinV staining by flow cytometry was measured after transduction with a shIRAK1-expressing lentiviral vector. (C-D) The indicated cell lines and primary MDS cells were transduced with shRNA-expressing lentiviral vectors (GFP+), sorted for GFP, and then plated in methylcellulose for progenitor colony formation. Colonies were scored 10-14 days after plating. (E) THP1 cells were transduced with a lentiviral vector containing a doxycycline(DOX)-inducible
promoter, which drives the expression of an IRAK1-targeting shRNA. Immunobloting and qRT-PCR confirm knockdown of IRAK1 upon addition of DOX. (F-G) MDSL cells transduced with the inducible shIRAK1 were transplanted into NSG mice (5 x 10^6/mouse). Half the mice received DOX-containing chow 7 days post engraftment. Human MDSL engraftment was determined by flow cytometry by measuring hCD45 in peripheral blood (F) and marrow (G). (Day 60: n = 7-8; Day 77: n = 7-8). (H) Spleen size is shown from a representative experiment at time of death. (I) Overall survival was determined for NSG (n = 12/group) mice transplanted with MDSL cells transduced with the inducible shIRAK1 (with or without DOX-containing chow). *, p < 0.05. Error bars represent +/- SD. See also Figure S5.
Figure 2.6. Integrated gene expression profiling of MDS cells following IRAK1 inhibition or deletion. (A) Microarray analysis was performed on MDSL cells transduced with shIRAK1 (or shRNA control) or treated for 48 hr with IRAK-Inh (or DMSO). GSEA was performed for both experiments in order to determine an overlapping gene signature for IRAK1 depletion. (B) Shown are the three overlapping GSEA profiles (from the top 10) generated from shIRAK1 and
IRAK-Inh MDSL gene signatures. (C) Shown are enriched GO pathways generated by Toppgene for shIRAK1 and IRAK-Inh MDSL cells. P values are shown only for the top GO pathway in each group. (D) BCL2 mRNA expression in MDSL treated with IRAK1 inhibitor (10 µM) or transduced with shIRAK1 adapted from the microarray analysis. Error bars represent +/- SD. (E) MDSL cells were treated with IRAK1 inhibitor (10 mM) or shIRAK1 expression induced with DOX for 24hr. Immunoblot analysis was performed to measure BCL2-family protein levels. (F) Densitometric values for BCL2 protein relative to GAPDH are summarized for the indicated cell lines treated with 10 µM IRAK-Inh. See also Figure S6.
Figure 2.7. Combined IRAK1 and Bcl-2 inhibition provides a collaborative cytotoxic effect against MDS and AML cells. (A) Viable cell growth of the indicated cell lines was assayed by trypan blue exclusion in the presence of IRAK-Inh (10 µM), ABT-263 (0.1 µM) or with the
combination of both drugs. (B) AnnexinV/7AAD staining by flow cytometry after 48hr treatment with IRAK-Inh (10 µM), ABT-263 (0.1 µM) or the combination of both drugs. (C) The indicated cell lines were evaluated for colony formation in methylcellulose in the presence of IRAK-Inh (10µM), ABT-263 (0.1µM) or the combination of both drugs. (D-F) Marrow cells from MDS (MDS-02) and AML patients (AML-01 and AML-02) were plated in methylcellulose containing IRAK-Inh (10 µM), ABT-263 (0.1 µM), or the combination of both drugs. (G) As in Figure 4A, overall survival was determined for NSGS mice transplanted with MDSL cells treated with IRAK-Inh, ABT-263, or with the combination of both drugs for 72 hrs. To the right, P-values are shown for the various experimental combinations. *, p < 0.05; **, p < 0.01; ***, p < 0.001. Error bars represent +/- SD. See also Figure S7.
Figure 2.S1, related to Figure 1. Deregulation of IRAK1 in MDS by miR-146a. (A) RNA isolated from MDS patient marrow cells (n = 20) was evaluated for IRAK1 mRNA. Shown is IRAK1 mRNA from MDS patients relative to the median expression from control marrow cells (n = 10; dashed line). MDS patients were divided into low (below 1.0) and high (above 1.0) IRAK1
expression. (B) Overall survival was determined for the MDS patients according to high and low IRAK1 expression. (C) IRAK1 mRNA (qRT-PCR), IRAK1 protein (immunoblot), and miR-146a (qRT-PCR) were determined for the indicated cells and normalized to normal mononuclear cells (MNC). Error bars represent +/- SD. (D) MDSL cells were transduced with a miR-146a decoy (pGK-GFP) as previously described (119). Endogenous miR-146a expression was reduced by ~75% (not shown). Expression of IRAK1 and TRAF6 was determined by immunoblotting and compared to vector-transduced cells. (E-F) Total (E) and phosphorylated (F) IRAK1 was quantitated and compared to miR-146a expression in the indicated cells. (G) IRAK1 and miR-146a expression was compared in MDS patient marrow cells (n = 20).
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**Table 2.S1**, related to Figure 1. MDS and AML patient characteristics
Figure 2.S2, related to Figure 2. IRAK-Inh selectively targets IRAK1 in MDS/AML cells and its effects are partially rescued by overexpression of TRAF6. (A) Protein lysates from MDSL cells treated with IRAK-Inh (µM) for 24 hrs were evaluated by immunoblotting for kinases that can be targeted by IRAK-Inh in non-hematopoietic cells and at higher concentrations: IRAK1 (IC50 = 0.3 µM); IRAK4 (IC50 = 0.2 µM); pp60Src (IC50 > 10 µM); and Lck (IC50 > 10 µM) (Powers et al., 2006). Densitometric values for phosphorylated relative to uns phosphorylated proteins and GAPDH are shown to the right. (B) THP1 cells were
transduced with empty vector-, IRAK1-, or TRAF6-containing LeGO-iG2 lentiviral vectors.

Immunoblots were carried out to confirm IRAK1 and TRAF6 overexpression (C) and to examine phospho-IRAK1 and GAPDH when treated with IRAK-Inh (µM). Densitometric values for pIRAK1 relative to GAPDH are shown below and normalized to 0 µM.
Figure 2.S3, related to Figure 3. Effects of IRAK-Inh on primary AML and normal CD34+ cells. (A) Primary AML cells were cultured in the presence of 10 µM IRAK-Inh for 48 hr and assayed for apoptosis by 7AAD and Annexin V staining. Protein lysates from MDSL cells treated with IRAK-Inh (µM) for 24 hrs were evaluated by immunoblotting for kinases that can be targeted by IRAK-Inh in non-hematopoietic cells and at higher concentrations: IRAK1 (IC50 = 0.3 µM); IRAK4 (IC50 = 0.2 µM); pp60Src (IC50 > 10 µM); and Lck (IC50 > 10 µM) (129). Densitometric values for phosphorylated relative to unphosphorylated proteins and GAPDH are shown to the right. (B) THP1 cells were transduced with empty vector-, IRAK1-, or TRAF6-
containing LeGO-iG2 lentiviral vectors. Immunoblots were carried out to confirm IRAK1 and TRAF6 overexpression (C) and to examine phospho-IRAK1 and GAPDH when treated with IRAK-Inh (µM). Densitometric values for pIRAK1 relative to GAPDH are shown below and normalized to 0 µM. (D-E) TRAF6-expressing cells were treated with IRAK-Inh and assessed for cell viability (D; trypan blue exclusion) and colony formation in methylcellulose (E). *, p < 0.05. Error bars represent +/- SD.
Figure 2.S4, related to Figure 4. Characterization of an MDSL xenograft model. (A) MDSL cells ($5 \times 10^6$) were transplanted into NSG mice. At time of disease, bone marrow (BM), spleen (SP), and blood (PB) were collected and analyzed for engraftment. In addition, BM, SP, and PB were stained with Wright-Giemsa. Scale bar, 5 µm. (B) Blood counts performed at time of death
for NSGS mice (n=3) indicate pancytopenia. As a control, sub-lethally (2.5Gy) irradiated NSGS mice were transplanted with normal Human umbilical cord blood (UCB) CD34+ cells and blood counts measured at 10 weeks post-transplant (n=8). (C) Bone marrow cellularity was determined for healthy NSG mice (HC) (n = 6), irradiated mice transplanted with normal CD34+ cells (n=5), and irradiated mice transplanted with MDSL cells (n = 5). Cellularity for mice transplanted with MDSL cells was determined when mice became moribund. (D) Marrow engraftment over time was determined at the indicated times by performing a femoral aspirate and calculated by measuring human CD45+CD33+.
Figure 2.S5, related to Figure 5. Lentiviral-mediated knockdown of IRAK1 mRNA and protein. (A) The MDSL cell line and CD34+ cord blood cells were transduced with pLKO.1 lentiviral vectors containing two independent shRNAs targeting IRAK1. IRAK1 mRNA levels were quantified by qRT-PCR at day 4 post-transduction. (B) Immunoblot assays were performed to quantify IRAK1 protein expression levels following lentiviral transduction of THP1 cells with either empty vector or two independent shRNAs targeting IRAK1. (C) Colony forming assays were carried out on MDSL cells transduced with pLKO.1 empty vector, or IRAK1-targeting shRNA containing vectors. (D) Domain architecture of doxycycline (DOX)-inducible TRIPZ shIRAK1 lentiviral construct. TRE, Tet-inducible promoter; RFP, red fluorescent protein; UBC, promoter. The shIRAK1 hairpin targets the 3-UTR of IRAK1. (E) Viable cell growth of the
TF-1 cell line transduced with TRIPZ shIRAK1, in the presence of absence of DOX. (F) Apoptosis was evaluated by flow cytometry of TF-1 cells transduced with TRIPZ shIRAK1 48hrs after addition of DOX. (G-H) TF1 expressing the TRIPZ shIRAK1 were transduced with vector (pLeGO-iG2) or IRAK1 cDNA that is resistant to the shIRAK1 hairpin (lacks the 3'UTR hairpin binding site). Following transduction and sorting (GFP+), cells were treated with DOX or vehicle control for 5 days and analyzed for IRAK1 expression by immunoblotting (G) and for AnnexinV staining (H). The IRAK1 immunoblot is shown after a short and long exposure. (I) Flow cytometric analysis of bone marrow (BM), spleen (SP) and peripheral blood (PB) of moribund NSG animals injected with TRIPZ shIRAK1-transduced MDSL. Flow plots were initially gated on viable, and then hCD45+ cells. Increase in RFP indicates expression of shIRAK1 in vivo. (J) Parental MDSL cells (1 x 10^6) were transplanted into irradiated NSG mice and administered doxycycline (DOX). *, p < 0.05. Error bars represent +/- SD. cells. *, p < 0.05.
Figure 2.S6, related to Figure 7. IRAK-Inh and ABT-263 collaborate to induce cytotoxicity.

(A) MDSL cells were treated with increasing concentrations of ABT-263 (0, 0.01, 0.1, and 1.0 µM) and IRAK-Inh (0, 0.1, 1.0, 5.0, and 10 µM) alone or in combination for 72 hrs. Surviving fraction of MDSL cells was determined by AnnexinV/PI staining and the live fraction values are shown inside the box. (B) Cells were treated with 0.1 µM ABT-263 and increasing concentration of IRAK-Inh. Note the cooperative cytotoxic effect of 0.1 µM ABT-263 with increasing concentrations of IRAK-Inh. (C) Cells were treated with 10 µM IRAK-Inh and increasing
concentration of ABT-263. Note the cooperative cytotoxic effect of 10 µM IRAK-Inh and 0.1 µM ABT-263. Dotted line represents the dose at which 50% of cells are alive for ABT-263 and in combination with IRAK-Inh.
Supplemental Experimental Procedures

Cell lines and CD34^+ cells
Acute myeloid leukemic cell lines, HL60, THP1, and TF-1 were purchased from the American Type Culture Collection. MOLM13 were purchased from AddexBio. The myelodysplastic cell line, MDS-L, was provided by Dr. Kaoru Tohyama (Kawasaki Medical School, Okayama, Japan) (155). Cell-lines were cultured in RPMI 1640 medium with 10% FBS and 1% penicillin-streptomycin. Additionally, both the MDSL and TF-1 cell lines were cultured in the presence of 10 ng/mL human recombinant IL-3 (Stemcell Technologies). Human CD34^+ umbilical cord blood (UCB) and adult marrow-derived mononuclear cells were obtained from the Translational Research Development Support Laboratory of Cincinnati Children’s Hospital under an approved Institutional Review Board protocol. Human CD34+ UCB cells and primary MDS/AML cells were maintained in StemSpan Serum-Free Expansion Media (Stemcell Technologies) supplemented with 10 ng/mL of recombinant human stem cell factor (SCF), Flt3 ligand (Flt3L), thrombopoietin (TPO), IL-3, and IL-6 (Stemcell Technologies).

Reagents
The IRAK1 inhibitor (IRAK1/4 inhibitor or IRAK-Inh; Amgen Inc.) was purchased from Sigma-Aldrich (I5409). Lipopolysaccharide was purchased from InvivoGen (TLRL-PEKLP9). ABT-263 was purchased from Chemietek (CT-A263) (176). Recombinant human IL1-β was purchased from PeproTech (200-01B). LeGO-iG2 lentiviral vectors for expression of IRAK1 and TRAF6 cDNAs are described previously (177).

Survival analysis and growth curves
Annexin V analysis was carried out as previously described (Fang et al., 2012). Cells were stained after either drug treatment or lentiviral transduction with Annexin V (eBioscience) and
propidium iodide (Sigma- Aldrich), or 7AAD (eBioscience) according to the manufacturer’s instructions. Analysis was performed using BD FACSCalibur or FACSCanto flow cytometer with either CellQuest or Diva software. For in vitro growth assays, cell expansion in liquid culture was determined based on trypan blue exclusion using an automated cell counter (BioRad TC10). For experiments beyond 48 hrs, cells were replenished with fresh media and drug every second day. For primary patient marrow cells, cell were treated with a single inhibitor dose and counted 24 and 48 hours later.

**NF-kB DNA-binding assay**

Nuclear lysates were isolated from treated cells as previously described (178). NF-κB (p65) DNA binding was measured using an ELISA-based assay according to the manufacturer’s recommendations (KHO0371, Invitrogen)

**Immunoblotting and Immunoprecipitation**

Total protein lysates were obtained from cells by lysing the samples in cold RIPA buffer (50mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 and 0.1% SDS), in the presence of PMSF, sodium orthovanadate, protease and phosphatase inhibitors. After being re-suspended in RIPA cells were briefly sonicated. Protein concentration was evaluated by a BCA assay (Pierce). For immunoprecipitation, TRAF6 or IRAK1 antibodies (2 µg) were added to cell lysates (10 mg) for 3 h at 4°C and captured by the addition of Protein A/G Plus beads (sc-2003; Santa Cruz) as described before (178). The immune complexes were washed with lysis buffer followed by the addition of SDS sample buffer. The bound proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and analyzed by immunoblotting. Western blot analysis was performed with the following antibodies: TRAF6 (sc-7221; Santa Cruz), IRAK1 (sc-7883; Santa Cruz), ubiquitin (sc-8017; Santa Cruz), IKKb (2370; Cell Signaling Technology), GAPDH (5174; Cell Signaling Technology), p38 MAPK (9212; Cell
Signaling Technology), phospho-p38 MAPK (4631; Cell Signaling Technology), phospho-IKKα/β (2697; Cell Signaling Technology), phospho-IRAK1 (S376) (PAB0497; Abnova), phospho-IRAK1 (T209) (A1074; AssaybioTech), phospho-ERK (4695; Cell Signaling Technology), Lck (2752; Cell Signaling Technology), phospho-Lck (Tyr505) (2751; Cell Signaling Technology), IRAK4 (4363; Cell Signaling Technology), phospho-IRAK4 (Thr345/Ser346) (7652; Cell Signaling Technology), Src (2102; Cell Signaling Technology), Phospho-Src (Tyr416) (2113; Cell Signaling Technology), BCL-2 (2870; Cell Signaling Technology) and Actin (4968; Cell Signaling Technology).

qRT-PCR
Total RNA was extracted using Trizol Reagent (Life Technologies) and reverse transcription was carried out using Superscript VILO (Life Technologies). Quantitative PCR was performed with Taqman Master Mix (Life Technologies) for human IRAK1 (Hs01018347_m1), TRAF6 (Hs04185733_m1), GAPDH (Hs02758991_g1) on an Applied Biosystems StepOne Plus Real-Time PCR System.

shRNA-mediated knockdown of IRAK1
Lentiviruses were pseudotyped with VSV-G, produced by 293-FT cells, and concentrated by ultracentrifugation at 20,000rpm for 2 hours at 4°C. Cells at 1x10⁵ cells/mL were transduced with lentivirus at multiplicity of infection (MOI) of 0.5~1 and in the presence of 8 µg/mL of polybrene (No. TR-1003-G; Millipore). At 48 hours post-transduction, GFP positive cells were isolated by fluorescence-activated cell sorting (FACS). The pLKO.1 (OpenBiosystems) constructs were obtained from the Lentiviral core at CCHMC and used to express shCTL (empty or scrambled control) and shIRAK1. Puromycin resistance gene was replaced by green fluorescent protein (GFP). Two independent and validated pLKO.1-shIRAK1 constructs were obtained: TRCN0000000543 and TRCN0000000544. After validation, majority of experiments
were performed with clone TRCN0000000544. For inducible knockdown of IRAK1, we used the TRIPZ doxycycline (DOX)-inducible shRNA system (OpenBiosystems) expressing shIRAK1 clone V2THS-132369.

**Xenograftment of NOD/SCID-IL2Rγ (NSG) and NSG-hSCF/hGM-CSF/hIL3 (NSGS)**

MDSL cells (1 x10^6 – 5 x10^6) were injected into the tail veins of 8-week old sublethally irradiated (250 Gy) NSG or NSG animals engineered to express human SCF, GM-CSF, and IL3 (NSGS). Mice were monitored by performing complete blood counts (Drew Hemavet 950FS). Following red cell lysis, human MDSL cells were identified by hCD45-FITC staining. IRAK-Inh was diluted in DMSO (5 mM) and further dissolved in sterile phosphate buffered saline (PBS; pH 7.2). Animals were injected i.p. with 2.12 mg/kg IRAK-Inh 3x weekly.

**Microarray analysis**

MDSL cells were transduced with lentivirus targeting human IRAK1 or a non-targeting control. At 2.5 days post-transduction, GFP positive populations were isolated by flow cytometry. Total RNA was extracted and purified with Quick-RNA MiniPrep Kit (Zymogen). RNA quality was tested using the Agilent Bioanalyzer 2100 (Hewelett Packard). Total RNA was reverse transcribed and labeled, and hybridized onto the GeneChip Human Gene 1.0 ST Array (Affymetrix). Scanning was performed with the Affymetrix GeneChip Scanner 3000 7G and evaluated with the Genechip Operating Software (Affymetrix). Data mining was performed with GeneSpring software (Agilent). Gene set enrichment analysis was performed on a JAVA-based dataset supported by the Broad Institute (157). For evaluation of IRAK1 expression in MDS, previously published data sets were used (179, 180). All microarray data files have been deposited in the Gene Expression Omnibus (accession number GSE46346).
Chapter 3: Differential IRAK Signaling in Hematologic Malignancies

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Results and Discussion

Interleukin receptor-associated kinase (IRAK) family mediate signals downstream of various pathogen- and cytokine-responsive receptors (181, 182). IRAK proteins consist of four functionally and structurally related members (IRAK1-4). In the context of hematological disorders, IRAK1 and IRAK4 are the most widely studied (114), and, in humans, are both ubiquitously expressed (183). Under normal cellular conditions, MyD88 is recruited to activated Toll-like receptors (TLR) or Interleukin 1 receptor (IL1R) resulting in activation of IRAK4 and IRAK1 (184). Activated IRAK1/4 proteins then bind TRAF6 to mediate NF-κB signaling. Activating mutations of MyD88 or B-cell receptor result in chronic IRAK4 phosphorylation and downstream pathway activation in human B cell lymphoma, particularly in the activated B-cell-like (ABC) subset of diffuse large B cell lymphoma (DLBCL) (114, 185). Knockdown of MyD88, IRAK4, or IRAK1 abrogate NF-κB pathway activation and induce ABC DLBCL cell death (114). Interestingly, IRAK4 catalytic function is necessary for maintaining the viability of DLBCL cells, while the catalytic function of IRAK1 is dispensable (114). These critical observations strongly implicate the dependency of ABC DLBCL on IRAK4 function.

More recently, we have reported that IRAK1 exists in an activated state (e.g., constitutively phosphorylated on Threonine-209) in a large subset human Myelodysplastic Syndrome (MDS) and Acute myeloid leukemia (AML) samples (118). In addition, overexpression of TLR1, TLR2 and TLR6 has been reported in MDS, and MDS-associated mutations of TLR2 correspond with increased IRAK1 activation (186). MDS originates within the hematopoietic stem cell (HSC) compartment and manifests into a multilineage erythro/myeloid disease (35). Patients with MDS also have a proclivity to develop AML (35). Knockdown of IRAK1 in MDS marrow cells and in a panel of MDS/AML cell lines resulted in cell cycle arrest, apoptosis, and impaired leukemic progenitor function. To further validate these findings, we treated cells with an IRAK1/4 Inhibitor. Consistent with the knockdown experiments, IRAK1/4
Inhibitor impaired MDS/AML cell viability and progenitor function, which also coincided with reduced levels of phosphorylated IRAK1, but not IRAK4.

Given the importance of the IRAK1/IRAK4 complex in human hematologic malignancies, we decided to investigate the role of IRAK4 in MDS. To discern differences between the expression of IRAK1 and IRAK4, published microarray data from MDS CD34+ cells were examined (151). IRAK4 expression is extremely low (at the lower limit of detection) and not significantly different as compared to control CD34+ cells (P = 0.073) (Figure 3.1A). By comparison, IRAK1 is preferentially expressed in normal CD34+ cells and further overexpressed in a subset (~20%) of MDS patients (P = 0.033) (Figure 3.1A). To functionally evaluate the contribution of IRAK1 versus IRAK4 in MDS cells, we performed RNAi-mediated knockdown experiments. MDSL cells transduced with shRNA targeting IRAK1 or IRAK4 were first evaluated for RNA and protein knockdown. As shown in Figures 3.1B and C, shIRAK1 clone #17 and shIRAK4 clone #65 resulted in approximately 90% knockdown of the respected targets; therefore, these shRNA clones were selected for further validation. As extensively described in our recent report (118), knockdown of IRAK1 in an MDS cell line (MDSL) results in apoptosis (Figure 3.1D) and impaired progenitor function in methylcellulose (Figure 3.1D). In contrast, knockdown of IRAK4 did not contribute to significant cell death of MDS cells (Figure 3.1C). Furthermore, knockdown of IRAK4 in MDSL reduced progenitor function (P = 0.0013), but not as dramatically as seen with knockdown of IRAK1 (P = 0.0004) (Figure 3.1E). Under stimulated conditions or in DLBCL, IRAK4 phosphorylates IRAK1. Interestingly, knockdown of IRAK4 in MDS cells only minimally reduces phosphorylated IRAK1 levels (Figure 3.1F), suggesting that IRAK1 is activated by alternative mechanisms in MDS. These findings reveal differences in IRAK1 versus IRAK4 dependency in MDS.

IRAK1 and IRAK4 are related kinases that function within the innate immune pathway. However, their roles in myeloid versus lymphoid malignancies appear to be distinct. The work by Dr. Staudt and colleagues has clearly established a critical role of IRAK4 in DLBCL and the
efficacy of targeting IRAK4 using an IRAK1/4 inhibitor (114). We propose that IRAK1, but not IRAK4, is essential in the pathogenesis of MDS/AML, and that small molecules selectively targeting IRAK1 may be therapeutically beneficial in MDS/AML. In conclusion, the innate immune pathway involving IRAK1 and IRAK4 signaling is important in the pathogenesis of hematologic malignancies, but their individual contribution is lineage and/or disease specific. As such, further research to discern the individual contribution of IRAK1 and IRAK4 to hematologic malignancies is warranted. Nevertheless, development of next generation IRAK inhibitors could be beneficial in both myeloid and lymphoid malignancies.
Figure 3.1. MDS cells depend on IRAK1, but less on IRAK4, for maintaining viability and progenitor function. (A) IRAK1 and IRAK4 expression is obtained from a gene expression study on CD34⁺ cells isolated from control and MDS marrows (IRAK1; p=0.036). (B) IRAK1 and IRAK4 mRNA knockdown was confirmed in MDSL cells by quantitative real-time polymerase chain reaction in cells expressing a control (shCTL), shIRAK1 (clone #17 and #18), or shIRAK4 (clone #63 or #65) lentiviral vector. (C) IRAK1 and IRAK4 protein knockdown was confirmed in MDSL cells by immunoblotting cells expressing shCTL, shIRAK1, or shIRAK4 lentiviral vector. (D) Annexin V staining by flow cytometry in MDSL cells was measured after transduction with
shIRAK1- or shIRAK4-expressing lentiviral vectors. (E) MDSL cells transduced with lentiviral vectors containing either shIRAK1 or shIRAK4 were plated at 2x10^4 cells/mL in methylcellulose. Colonies were counted 11 days after plating. (F) MDSL cells transduced with lentiviral vectors containing shIRAK4 or shCTL (same lysates as in C) were analyzed for pIRAK1 (at threonine-209) and total IRAK1. ns=nonspecific.
Chapter 4: An MDS xenograft model utilizing a patient-derived cell line

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Published in \textit{Leukemia}. 2013 Dec 11. [Epub ahead of print]. PMID: 24326684.
Results and Discussion

Animal models that faithfully recapitulate clinical features of Myelodysplastic Syndrome (MDS) have been difficult to establish. Myelodysplastic syndromes (MDS) are hematologic disorders associated with ineffective hematopoiesis, blood cytopenias, myeloid dysplasia, and an increased risk of acute myeloid leukemia (AML). Although transplantation of primary AML cells into immunocompromised mice has been met with much success, primary MDS patient samples exhibit poor engraftment with no evidence of disease in recipient animals (187-189). The engraftment of primary MDS samples is complicated further by frequent outgrowth of normal HSC clones (190). These challenges underscore the current limitations and inefficiencies of engrafting primary MDS cells. Recent improvements in engrafting primary MDS cells have been observed when transplanting immunophenotypically-defined HSC from the marrow of MDS patients or when co-injecting stromal cells engineered to produce non-crossreacting human cytokines (191, 192). Unfortunately, even with improved engraftment of primary MDS cells, mice do not succumb to features resembling human MDS, precluding the use of these models for preclinical testing. To circumvent the current limitations, we developed a model using immunocompromised recipient mice and a human MDS cell line (MDSL) derived from the non-leukemic phase of an MDS patient with refractory anemia-ringed sideroblasts (118, 155, 193). MDSL was derived as a subline of MDS92, and maintains factor dependency for cell growth, but has reduced differentiatitional capacity compared to MDS92 (194). Herein, we report the successful engraftment of MDSL cells into NOD/SCID-IL2Rg mice (NSG) and NSG-hSCF/hGM-CSF/hIL3 (NSGS) mice and reproducible development of disease, including cytopenias, clonal expansion, and host hematopoietic suppression. In addition, we show that the MDSL xenograft model is a useful tool for evaluating novel and existing therapeutics for MDS.

As reported for the original parental MDS92 line (195), MDSL cells have maintained cytokine dependence in vitro. Upon hIL-3 withdrawal, MDSL cells stop growing within 3 days.
and die within 5 days (Figure 4.1A). Given the challenges of engrafting primary human MDS samples, we sought to determine the engraftment and disease-initiating potential of MDSL. 1x10^6 MDSL cells were injected intravenously (i.v.) into 8-10 week old sublethally-irradiated NOD/SCID-IL2Rg (NSG) or NSG-hSCF/hGM-CSF/hIL3 (NSGS) mice, and monitored for evidence of hematopoietic malignancy. MDSL engraftment in NSGS or NSG resulted in a fatal hematologic disease associated with cytopenia, marrow failure, and organ infiltration, with a median survival of 24 and 70 days post-injection, respectively (Figure 4.1B). The shorter latency observed with the NSGS is attributed to the transgenic expression of three non-crossreacting human cytokines (SCF, GM-CSF, and IL3), which provide an enhanced milieu for the myeloid cell grafts (174). At the time of sacrifice, flow cytometric analysis revealed efficient MDSL engraftment of hCD45^+ cells in the bone marrow (BM), spleen (SP) and peripheral blood (PB) of both NSG and NSGS recipient mice (Figure 4.1C). Interestingly, conditioning NSG or NSGS mice with sub-lethal radiation was not a requisite for MDSL cell engraftment, however non-irradiated recipients did exhibit delayed disease latency (data not shown).

Intrafemoral BM aspirates of NSG at 53 days post-transplant revealed an average human MDSL (CD45^+CD33^+) graft of 2% total marrow (Figure 4.1D). However, at time of death (82 days post-transplant), MDSL cells progressively expanded to occupy ~60% of the total marrow (Figure 4.1D). BM cellularity was determined for MDSL- and CD34^+ cord blood-engrafted NSG mice 10 weeks post-transplant. Although MDSL cells gradually expanded over time, the total cellularity of MDSL-engrafted marrows was 70% less than CD34^+ cord blood-engrafted marrows (5 x 10^5 versus 22 x 10^5 cells/femur) (Figure 4.1E). Immunohistochemical examination of BM from MDSL engrafted NSG mice confirmed a hypocellular marrow, while control CD34^+ cell-engrafted mice exhibited normal BM cellularity (Figure 4.1F). In MDSL-engrafted mice, we also observed bone remodeling at the endosteum (Figure 4.1F). These findings indicate that MDSL cells undergo clonal expansion while simultaneously displacing host mouse BM cells.
To examine subclonal selection following transplantation, MDSL cells were immunophenotypically evaluated for the expression of hematopoietic stem/progenitor and myeloid cell markers immediately prior to transplantation (Figure 4.1G) and 10 weeks post-transplantation (Figure 4.1H). Pre-transplant, MDSL cells were found to express hematopoietic stem/progenitor markers (CD34, CD117), as well as several myeloid cell markers (CD11b, CD38, CD13, CD33) (Figure 4.1G). By comparison, MDSL cells isolated from BM of NSG mice displayed similar immunophenotypic markers (Figure 4.1H). The morphology of MDSL cells was determined by Wright-Giemsa staining of cytospins and blood smears before and after transplantation (Figure 4.1I). The morphology of MDSL grown in vitro mirrored what was observed in the BM, SP and PB of xenografted recipient mice (Figure 4.1I). At both time points, MDSL cells displayed a dysmorphic and heterogeneous myeloid phenotype (Figure 4.1I). After in vivo expansion, MDSL cells did not acquire a blast phenotype as typified by many AML cell lines. Additionally, both immunodeficient mouse strains exhibited secondary transplantation of MDSL. Secondary transplants exhibited similar disease phenotype, and disease latency was not significantly different from primary transplantations (data not shown). Collectively, these observations suggest that minimal subclonal selection and immunophenotypic evolution occurs in MDSL xenografts.

We chose to further characterize NSG animals, since they presented with a predominant BM graft that might more closely represent human disease pathology in MDS. To investigate the consequences of MDSL xenografts on host mouse hematopoiesis, recipient NSG mice transplanted with MDSL or control CD34+ cells were monitored by complete blood count analysis. By 10 weeks, mice xenografted with MDSL cells exhibited reduced mouse red blood cells (P = 0.04), hemoglobin levels (P = 0.02), and platelet counts (P = 0.07) as compared to mice transplanted with CD34+ cells (Figure 4.1J). This observation suggests that the MDSL cell graft impairs mouse hematopoiesis resulting in anemia and thrombocytopenia.

Next we tested the MDSL xenograft model as a pre-clinical tool for examining drug
efficacy. Lenalidomide (LEN) is FDA approved for first-line treatment of low-risk del(5q) MDS (196), and MDSL cells have been reported to be sensitive to LEN in vitro (155). For these reasons, we believed LEN would be an ideal therapeutic agent to test our experimental system. Consistent with previous reports, in vitro treatment of MDSL with 10 µM LEN resulted in increased apoptosis (as measured by Annexin V/PI staining; Figure 4.2A) and reduced growth (as measured by trypan blue exclusion; Figure 4.2B). Furthermore, the effect of LEN on progenitor function was evaluated using a methylcellulose assay. MDSL cells formed 50% fewer colonies (P < 0.05) in methylcellulose when cultured with LEN (Figure 4.2C). To examine the effect of LEN in vivo, 1x10^6 MDSL cells were xenografted into NSG mice following sub-lethal irradiation. To mimic a similar dosing regimen as for MDS patients, NSG mice were given a nearly continuous 25 mg/kg of LEN for 7 weeks (Figure 4.2D). To ensure efficient MDSL engraftment, LEN administration commenced 10 days post-transplant. The MDSL cell graft (hCD45^+CD33^+) was monitored in the blood of NSG animals at day 55 and day 70-post transplant. At both time points the LEN-treated cohort exhibited reduced MDSL engraftment (Figure 4.2E). NSG mice transplanted with MDSL but receiving only vehicle control (DMSO) appeared moribund between 40-75 days (Median survival: 48 days; Figure 4.2F). In contrast, NSG treated with LEN achieved significantly longer overall survival (Median survival: 82 days; Figure 4.2F). Taken together, these data suggest that LEN is capable of suppressing the MDSL clone both in vitro and in the in vivo setting provided by our xenograft model.

In the present study, we examined the ability of NSG and NSGS mice to support the growth of an MDS patient-derived cell line. Phenotypic characterization of MDSL has revealed that they retain the dysplastic, non-blast like morphology in vitro and in vivo. Unlike AML cell-line xenografts, the MDSL graft does not undergo clonal selection, and does not exhibit shortened disease latency, following secondary transplantation. Although there are notable limitations of using a single cell line-based xenograft system to extrapolate human disease and preclinical assessment of therapeutics, several important advantages exist. Mice transplanted with MDSL
exhibit efficient engraftment, which permits serial tracking of the MDS clone, and develop a fatal disease. Importantly, certain hematopoietic parameters of MDSL-xenografted mice, namely anemia and thrombocytopenia, mirror features associated with human MDS.

Following transplantation of MDSL, both NSG and NSGS animals displayed human cell grafts, however the disease features occurred in NSGS animals with a significantly shortened latency. The accelerated disease in NSGS xenografted mice suggests that the transgenic expression of hGM-CSF, hSCF and hIL3 enhances growth and survival of the MDSL cells in vivo. Given that both NSG and NSGS mice permit efficient engraftment of MDSL cells into the marrow, yet with different kinetics, this model provides a platform to study niche interactions and the inter-relationship between MDS cells and the host marrow environment. Interestingly, the hypoplastic marrow subtype of MDS (~15% of all cases) is typically associated with immune-mediated pathogenesis, with increased levels of pro-inflammatory cytokines (197). Our MDS model generates a hypocellular marrow environment, and thus provides an opportunity to potentially examine this disease variant in greater detail.

Additionally, MDSL cells are capable of engrafting into immunodeficient recipients without the use of radiation, thus making these animals amenable to treatment with traditional combination chemotherapies. Evaluating chemotherapies within this model system would be otherwise impossible if radiation pre-conditioning was required for MDSL engraftment (198). In particular, the short disease latency of this model provides an opportunity for a timely experimental readout in pre-clinical MDS studies. Moreover, the anemia and thrombocytopenia exhibited by this model provide readily measurable secondary endpoints when assessing the disease-modifying activity of novel agents in vivo. In summary, given the faithful recapitulation of an MDS-like phenotype and the rapid experimental disease readout, we believe that MDSL xenografts will become an important tool in studying the activity of novel agents designed for MDS.
Acknowledgements

This work was supported by Cincinnati Children’s Hospital Research Foundation, American Society of Hematology (ASH), National Institute of Health (RO1HL111103), and Department of Defense grants to D.T.S, and a NIH/NCRR Institutional Clinical and Translational Science Award (1UL1RR026314-01) to J.C.M. D.T.S. is an ASH Scholar and J.C.M. is a Leukemia and Lymphoma Scholar. The umbilical cord CD34⁺ samples were received through the Normal Donor Repository in the Translational Core Laboratory at Cincinnati Children's Research Foundation, which is supported through the NIDDK Center's of Excellence in Experimental Hematology (P30DK090971). We thank the Mt. Auburn Ob-Gyn associates and delivery nursing staff at Christ Hospital (Cincinnati, OH) for collecting cord blood (CD34⁺) samples from normal deliveries. We thank Jeff Bailey and Victoria Summey for assistance with transplantations and xenotransplantations (Comprehensive Mouse and Cancer Core). MDSL cells were kindly provided by Dr. Kaoru Tohyama and are made available upon request (ktohyama@med.kawasaki-m.ac.jp or Daniel.Starczynowski@cchmc.org). The authors declare no competing financial interests.

Experimental Procedures

Cell lines and CD34⁺ cells

The myelodysplastic cell-line (MDSL) was previously described (155, 193, 195). MDSL were cultured in RPMI 1640 medium with 10% FBS, 1% penicillin-streptomycin, and 10 ng/mL human recombinant IL-3 (StemCell Technologies). Human CD34⁺ umbilical cord blood cells were obtained from the Translational Research Development Support Laboratory of Cincinnati Children’s Hospital under an approved Institutional Review Board protocol.
**Growth curves, progenitor assays, and survival analysis**

For in vitro growth assays, cell expansion in liquid culture was measured by trypan blue exclusion using an automated cell counter (BioRad TC10). For progenitor assays, cells were plated in methylcellulose (1x10^4 cells/plate) (04434; Stemcell Technologies) containing either DMSO, or 10 mM LEN, and evaluated for colony formation after 14 days (as previously described (118, 177). For cell survival, apoptosis was measured by AnnexinV staining, as described previously (199). The Annexin V antibody was obtained from eBioscience (17-8007-74).

**Reagents**

Lenalidomide (LEN) was purchased from LC Laboratories (L-5499). Antibodies used for immunophenotyping MDSL were ordered from BD Pharmingen: CD33PE (555450), CD13PE (555394), CD16PE (555407), CD117PE (555714), CD34APC (555824), CD36APC (550956), CD11bAPC (550019), CD19APC (555415), CD38PE (553764); and, Miltenyi Biotec: CD133PE (130-098-046).

**Xenograft of NOD/SCID-IL2Rg mice (NSG) and NSG-hSCF/hGM-CSF/hIL3 (NSGS)**

All animal experiments were performed in adherence to protocols approved by the Institutional Animal Care and Use Committee of Cincinnati Children’s Hospital Medical Center. MDSL cells (1 x 10^6) were injected into the tail veins of 8-week old sublethally-irradiated (2.5 Gy) NSG or NSG animals engineered to express SCF, GM-CSF, and IL3 (NSGS), as previously described (174). Mice were monitored by performing complete blood counts (Drew Hemavet 950FS). Following red cell lysis, human MDSL cells were identified by flow cytometric staining (hCD45^+CD33^+). In vivo delivery of LEN was adapted from previous reports (200). Briefly, LEN was diluted in DMSO to a 5 mM stock and further dissolved in sterile phosphate buffered saline (PBS; pH 7.2) and 0.02% Tween 20. Animals were injected i.p. with 25 mg/kg LEN 5x/week.
Statistical Analysis

Results are depicted as the mean ± standard error of the mean. Statistical analyses were performed using Student’s $t$-test. GraphPad Prism (v6, GraphPad) was used for the generation of survival curves and statistical analysis.
Figure 4.1. Development and characterization of a MDSL xenograft model. (A) Growth curves of MDSL cultured with or without hIL-3 (10 ng/mL). Cell viability was determined by trypan blue exclusion at the indicated time points. (B) NSG and NSGS animals were sub-lethally
irradiated and injected i.v. with 1x10^6 MDSL and evaluated for survival. **(C)** MDSL cells (1 x10^6) were transplanted into NSG and NSGS mice. At time of disease, bone marrow (BM), spleen (SP), and peripheral blood (PB) were collected and analyzed for engraftment by flow cytometry. **(D)** Serial marrow aspirates were performed on MDSL-engrafted (n=4) mice at days 53 & 82 post injection and examined via flow cytometry to assess human and mouse cell grafts. **(E)** BM cellularity was examined for NSG mice xenografted with either human CD34^+ umbilical cord blood (UCB) cells (n=5) or with MDSL cells (n = 5). **(F)** Hematoxylin and eosin staining was performed on paraffin-embedded bones from MDSL- and CD34^+ UCB-engrafted NSG mice. **(G-H)** MDSL cells were examined by a panel of flow cytometry antibodies prior to injection into mice (In vitro, G) and following harvest from moribund animals (Ex vivo, H). **(I)** Wright-Giemsa staining of MDSL cultured in vitro, and cells harvested from the BM, SP and PB of NSG animals. Arrows indicate MDSL cells. **(J)** Complete blood counts performed at time of death for NSG animals (n=3) xenografted with MDSL cells (at time of death) or CD34^+ UCB cells (at 10 weeks post-transplant (n=8)). *, P value < 0.05; ***, P value < 0.001
Figure 4.2. The MDSL xenograft model as a pre-clinical tool for drug evaluation. (A) Annexin V/PI staining of MDSL cultured in DMSO or 10 mM LEN was determined after 48hr. (B) Viable cell growth of MDSL was assayed by trypan blue exclusion in the presence of DMSO or LEN (10 mM). (C) MDSL cells were evaluated for colony formation in methylcellulose containing either DMSO or LEN (10 mM). (D) Sub-lethally irradiated NSG recipient animals were injected with 1x10^6 MDSL; after 10 days mice were subjected to 7 cycles of DMSO or LEN (25 mg/kg) by I.P. administration. (E) NSG animals were bled at day 55 and day 70. Red blood cells were lysed and cells were stained with mCD45-APC-Cy7, hCD45-FITC and CD33-PE and examined by flow cytometry. (F) Kaplan-Meier analysis of MDSL xenograft-bearing NSG mice administered I.P. with LEN. *, P value < 0.05; **, P value < 0.01
Chapter 5: FLT3 and IRAK kinase inhibitors for Acute Myeloid Leukemia

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Currently in preparation.
Abstract

FLT3 is a type III receptor tyrosine kinase (RTK) necessary for early hematopoietic development. Mutations in FLT3 are among the most common genetic lesions in Acute Myeloid Leukemia (AML). Approximately 20-25% of AML patients have an internal tandem duplication (ITD) within the FMS-like region of the kinase, and another 5-10% have mutations present in the activation loop, typically at aspartate 835 (D835) (201). The mutational frequency of FLT3 makes it an attractive therapeutic target in AML. First generation small-molecule kinase inhibitors of FLT3 evoked transient marrow responses and were largely ineffectual owing to poor pharmacokinetic/pharmacodynamic (PK/PD) properties. Currently, quizartinib is the most advanced FLT3 inhibitor and has demonstrated clinical activity. Unfortunately, patients who respond to quizartinib often have incomplete hematologic and/or platelet recoveries, and exhibit short-lived bone marrow responses (202). Current clinical evidence suggests that the best use for quizartinib may be combining with traditional chemotherapies and/or using this agent as a bridge to hematopoietic stem cell transplant (HSCT). Thus there still is an urgent unmet need for agents that are capable of providing durable, long-term responses. Our work has uncovered the overexpression and activation of the serine/threonine kinase, IRAK1, in AML. Furthermore, pharmacological inhibition of IRAK1 results in AML cell apoptosis. We therefore hypothesized that a pharmacological strategy employing combined FLT3 and IRAK inhibition would offer increased therapeutic potency and benefit, in the context of FLT3-mutant AML. Recent evidence delineating the activation of NF-κB within the context of FLT3-mutated AML subtypes provides additional scientific rationale for this approach (203-208). Herein, we report the structure and cellular activity of four imadizo[1,2-a]pyridine tool compounds that function as dual IRAK/FLT3 inhibitors. Three of the imadizo[1,2-a]pyridine analogues were 30- to 1300-fold more potent than quizartinib in FLT3-ITD cellular assays. Our results suggest that co-inhibition of IRAK and FLT3 may represent an attractive therapeutic strategy in FLT3-mutant AML.
Results

Structure and activity of dual FLT3/IRAK kinase inhibitors

We hypothesized that building in IRAK inhibitory activity into FLT3-targeted small molecules would provide enhanced activity against FLT3-mutant AML cells. To test this hypothesis, using structure-activity relationships our collaborator (Dr. Craig Thomas, NIH/NCATS) synthesized several imadizo[1,2-a]pyridine analogues from NCGC00249836 (NCGC00262331, NCGC00241410, NCGC00262376, NCGC00262327) (Figure 5.1). Each of the four analogues exhibited low nM activity against the enzymatic activity of FLT3, IRAK1 and IRAK4 (Figure 5.2).

Cellular activity of dual FLT3/IRAK kinase inhibitors

In order to determine the cellular potency of the imadizo[1,2-a]pyridine FLT3/IRAK tool inhibitors, we utilized BaF3 cells transduced with FLT3, FLT3-ITD, or FLT3-ITD D835Y. Although NCGC00241410, NCGC00262376, and NCGC00262327 exhibited low nM EC50s against FLT3- and FLT3-ITD-transduced BaF3, quizartinib was approximately 9- to 24-fold more potent in these cell types. However, NCGC00262376, and NCGC00262327 were approximately 4- to 5-fold more potent than quizartinib against BaF3 transduced with FLT3-ITD D835Y.

In order to examine the cellular potency of these inhibitors in a more relevant physiological setting, we engineered human CD34+ cord blood cells to express MLL-AF9 (MA9) and either FLT-ITD or NRas G12D. Although MA9 expression is not sufficient for leukemic transformation, expression of FLT-ITD or NRASG12D converts the MA9 cells to overt AML(198). FLT3-ITD-expressing MA9 cells exhibited exquisite sensitivity to FLT3/IRAK-targeted agents, while NRas G12D-expressing MA9 cells were refractory (Table 5.2). NCGC00241410, NCGC00262376, and NCGC00262327 exhibited low nM EC50s in FLT3-ITD-expressing MA9 cells, that were 30- to 1300-fold more potent than quizartinib. Interestingly, quizartinib had a 1.2-
1.5 $\mu$M EC50 in the human FLT3-ITD cells, which contrasts with 2.9 nM EC50 achieved in mouse FLT3-ITD BaF3 cells.

The 2-acyl-aminobenzimidazole, NCGC00241411, was included because it exhibited highly selective activity against IRAK4 (2.5 nM) and IRAK1 (38.4 nM) without inhibiting FLT3 (>10,000 nM) (Table 5.1). The cellular activity of this compound was markedly lower than other analogues, suggesting that FLT3 inhibitory activity is necessary to obtain potency against FLT3 dependent cells.

NCGC00241410, NCGC00262376, and NCGC00262327 were selected for further evaluation based on EC50 values. In order to examine their effect on AML progenitor activity, we utilized colony-forming cell assays. At 100 nM NCGC00262327 potently inhibited colony formation in FLT3-ITD MA9 cells, and colony inhibition could be rescued through exogenous addition of FLT3L (Figure 5.2). Additionally, NCGC00241410, NCGC00262376 were incapable of inhibiting colony formation at 100 nM. The colony formation of MA9 cells transduced with NRas G12D was not affected by these analogues, suggesting that these agents are only effective against leukemic clones which are driven primarily through FLT3 signaling.
Figure 5.1. Chemical structures of FLT3/IRAK inhibitors. The structures of five imadizo[1,2-a]pyridine analogues (NCGC00249836, NCGC00262331, NCGC00241410, NCGC00262376, NCGC00262327) and a 2-acyl-aminobenzimidazole (NCGC00241411) are shown. NCGC00241411 was utilized because of its highly selective against IRAK kinase activity. NCGC00249836 served as a imadizo[1,2-a]pyridine scaffold, which was used in further SAR studies, resulting in the synthesis of NCGC00262331, NCGC00241410, and NCGC00262376, NCGC00262327.
Table 5.1. Biochemical IC50 determination for FLT3/IRAK inhibitors. IC50 data were obtained through the Reaction Biology kinase assay, which utilized $^{33}$P-ATP and ion exchange chromatography to determine enzymatic activity.
### Table 5.2. Summary of FLT3/IRAK inhibitor EC50 determination.
The murine BaF3 pro-B cell line was transduced with retroviral vectors containing wild-type FLT3, FLT3-ITD, or FLT3-ITD with a single amino acid substitution D835Y. Parental BaF3 cells were grown in the presence of 10 ng/mL IL3. Two human umbilical cords (clone .3 and clone .6) underwent CD34⁺ selection via autoMACS cell separation prior to retroviral transduction with constructs containing MLL-AF9 and FLT3-ITD or NRas G12D. Both human and mouse cells were seeded in triplicate for 72 hr in 96-well format with presence of NCGC drug or DMSO to determine a 10-point dose response curve. Raw data was collected using a GloMax luminometer and the CellTiter-Glo luminescent Cell Viability Assay (Promega). EC50 values were obtained using nonlinear regression.
Figure 5.2. NCGC00262327 impairs AML progenitor function and exogenous FLT3L is capable of rescuing colony formation. (A) Human MLL-AF9 and FLT3-ITD or NRas G12D transduced AML cells were plated into methylcellulose (H4434, StemCell Technologies) at 1 x $10^5$ cells/mL in the presence of NCGC compounds or DMSO as indicated. (B) Same experiment as (A) except 10 ng/mL exogenous FLT3L was added to the methylcellulose during plating.
Chapter 6: Discussion, Implications, and Future Directions

Development of novel therapies for MDS

Currently, the only curative treatment for MDS patients is allogeneic HSCT, and unfortunately, patients are ineligible for HSCT after the age of 65 (209). Although reduced-intensity conditioning (RIC) regimens have been effective in reducing transplant-related mortality (210), only one third of patients receiving HSCT are cured by treatment (211-213); remaining patients suffer from transplant-related mortality or disease relapse. Besides transplantation, the only other therapeutic options shown to offer a survival benefit are the DNA methyltransferase inhibitor (DMTI) hypomethylating agents, 5-azacytidine (AzaC) and decitabine. These drugs have been shown to decrease the risk of leukemic transformation, and in a subset of high-risk patients, improve survival (214-216). However, the survival benefit of these drugs is still marginal, i.e. 24 vs. 16 months for patients randomized to AzaC compared to those in the control arm. Serial measurement of MDS-initiating progenitor cells from high-risk MDS patients treated with AzaC has demonstrated transient reductions in the CD34+ MDS progenitor cell population in patients achieving a CR/CRi (46). However, for many patients receiving hypomethylating agents, hematologic responses are short-lived. Ultimately, hypomethylating agents are ineffective at completely eliminating MDS progenitor cells. Namely, patients exhibit rapid expansion of the remaining MDS progenitor population prior to morphological relapse (46).

Unlike high-risk MDS, low-risk MDS patients often receive supportive care in combination with blood transfusions with the goal of providing hematologic improvement. Lenalidomide, a thalidomide analogue, is FDA-approved for the treatment of patients with transfusion-dependent anemia due to low-risk MDS associated with del(5q) (196). In a randomized, multicenter registrational trial lenalidomide was shown to induce transfusion
independence, and hematologic improvement in a subset of patients. According to a retrospective analysis of the Spanish MDS Registry database, lenalidomide-treated patients are not conferred survival or leukemia-free survival benefits (217), although more comprehensive studies are required to investigate the long-term effects that lenalidomide may have on survival and leukemic progression. Longitudinal examination of MDS patient marrow over the course of lenalidomide treatment reveals that the del(5q) MDS clone is resistant to treatment, even during a time of complete clinical and cytogenetic remission (159). The persistence of these clones ultimately leads to disease relapse and, in some cases, progression to AML. Thus a new generation of drugs that effectively target, and eradicate the MDS-initiating clone, will prove to be more effective therapies and likely provide a significant survival advantage to patients.

TLR and IL1R superfamily signaling has a critical role in regulating normal hematopoietic stem cell differentiation (218, 219), and dysregulation of certain pathway components can result in the initiation of myeloid malignancies, including AML (220-224), myeloproliferative disorders (120, 121), BMF (225) and MDS (119). At the outset of this thesis we posited that certain mediators of TLR/IL1R signaling would be suitable therapeutic candidates for MDS. One such mediator, IRAK1, emerged as a top candidate. IRAK1 is a serine/threonine kinase that relays signals from TLR/IL1R to NF-κB. Following receptor engagement, via death-domain interactions, IRAK1 is recruited to the Myddosome, a multimeric signaling complex composed of MyD88, IRAK2 and IRAK4 (79). IRAK1 is initially activated by phosphorylation at T209, and mutation of this residue prevents IRAK1 kinase activity (55). After becoming phosphorylated, IRAK1 associates with the TRAF6 signaling complex and undergoes K63-linked polyubiquitination. The interaction between IRAK1 and TRAF6 results in nuclear translocation of NF-κB, and positively regulates subsequent NF-κB target gene transcription. Interestingly, both TRAF6 and IRAK1 are validated targets of miR-146a. Deletion of miR-146a occurs in approximately ~80% of MDS with del(5q), and reduced expression of miR-146a is a common
feature among normal karyotype (NK) MDS (119). Germline knockout of miR-146a results in derepression of IRAK1 and TRAF6 protein, and a myeloproliferative phenotype in mice (120, 121). Similarly, when TRAF6 expression is enforced in mouse BM, animals develop BMF and AML (119). Thus miR-146a and TRAF6 represent two important players within the TLR/IL1R pathway, which function to drive MDS pathogenesis. Unfortunately, both of these factors are poorly tractable from a drug development standpoint. For example, miRNA replacement therapies are in nascent stages and presently face formulation and delivery challenges (226).

Similarly, targeting E3 ubiquitin ligases is a promising approach, however, beyond early prototype MDM2 inhibitors, very few other E3 ligase inhibitors have been reported (227). The paucity of E3 inhibitors speaks to the challenge of targeting, and selectively disrupting, the protein-protein interactions between E2 and selected E3 ubiquitin ligases. The lack of a suitable hydrophobic pocket in many E3 ligases at this protein-protein interface presents a significant medicinal chemistry challenge. Thus, like miR-146a, TRAF6 currently appears to be a difficult drug target. However, unlike both miR-146a and TRAF6, there are a number of tool compounds available to probe the biological function of IRAK1 in MDS (128-132, 136).

Although IRAK1 mRNA expression is higher in CD34+ BM from MDS patients when compared to healthy, age-matched controls, the limit of IRAK1 overexpression is approximately 2.5-fold. However, MDS patient BM cells exhibit marked overexpression of IRAK1 protein, when compared to healthy BM cells. This suggested post-transcriptional dysregulation of IRAK1 expression. As expected, we verified that IRAK1 protein expression is post-transcriptionally regulated by miR-146a in MDS cells (Figure 2.S1D). Thus IRAK1 expression is dysregulated transcriptionally, and post-transcriptionally in MDS. These initial findings implicated IRAK1 as a candidate target for disrupting TLR/IL1R signaling in MDS cells.

We found that inhibiting IRAK1, through either pharmacological or genetic approaches, resulted in delayed proliferation, cell cycle arrest, increased apoptosis, and impaired progenitor function in primary MDS BM cells, and MDS cell lines (Figure 2.3 and Figure 2.5A-D). These
observations were consistent with an integrative gene-expression analysis, demonstrating that IRAK1 regulates genes involved in survival, cell cycle, and proliferation (Figure 2.6C). The downstream signaling consequences of inhibiting IRAK1 involved decreased TRAF6 activation, and decreased canonical and non-canonical NF-κB activity (Figure 2.2). Enforced TRAF6 expression was sufficient to partially rescue the cellular effects of IRAK inhibition (Figures 2.S3H and 2.S3I), suggesting that IRAK inhibition may impact on additional downstream non-TRAF6 related molecules. A global analysis of serine- and threonine-phosphorylation sites in wild type and IRAK deficient MDS cell lines utilizing a phosphoproteomics approach would ostensibly define novel, relevant IRAK1 signaling targets and should be considered for future studies.

Subsequent pre-clinical target validation of IRAK1 required us to engineer a novel orthotopic xenograft model of MDS. Animal models that recapitulate features of MDS have been very difficult to establish. When xenografted into immunocompromised animals, primary MDS cells exhibit poor engraftment with no evidence of disease in recipient animals (187-189). Additionally, the MDS clone is frequently outgrown by normal HSCs present within the patient sample (190). These challenges highlight the current limitations of modeling MDS in vivo via engraftment of primary MDS cells. To circumvent these constraints we engineered an MDS xenograft model (Chapter 4) utilizing a human MDS cell line (MDSL) derived from the non-leukemic phase of an MDS patient with RARS (118, 155, 193). We utilized NSG and NSGS mice, which are the most immunocompromised strains currently available and are entirely depleted of B, T and NK cell activity (174). NSGS animals exhibited shortened latency, owing to the transgenic expression of human IL3, SCF and GM-CSF, which provide an enhanced milieu for myeloid cell expansion (174). Immunophenotypic characterization, and secondary transplants of ex vivo MDSL suggested that these cells had not undergone clonal selection or transformation to AML. For example, secondary MDSL transplants did not exhibit shortened disease latency and the immunophenotype of ex vivo MDSL cells mirrored that of primary cultures (Figure 4.1G). Since MDSL are derived from a low-risk MDS patient, lenalidomide,
which is FDA approved for low-risk del(5q) MDS (196), was chosen to test the utility of this model as a pre-clinical drug evaluator. MDSL cells have been reported to be lenalidomide sensitive in vitro (155) and this finding was reproducible in our hands (Figures 4.2A-C). By using a strategy to mirror the clinical lenalidomide treatment regimen within the MDSL xenograft model (Figure 4.2D), we found that lenalidomide was effective in reducing the MDSL cell burden and significantly increasing survival in NSG mice (Figures 4.2E and 4.2F). Therefore our MDSL xenograft model faithfully recapitulates MDS features, namely anemia and thrombocytopenia, and exhibits very short disease latency, which supports the timely evaluation of anti-MDS agents. MDSL do not undergo clonal evolution in vivo, this is demonstrated by retention of immunophenotype, morphology, and drug sensitivity; thus the MDSL xenograft model represents a bona fide MDS animal model.

With the MDSL xenograft model in hand we sought to examine the in vivo efficacy of IRAK1 inhibition. Treatment of MDSL with IRAK-Inh prior to engraftment into NSG and NSGS recipients resulted in reduced MDSL engraftment, delayed anemia and thrombocytopenia, and significantly increased survival (Figure 2.4). An in vivo RNAi-mediated approach, utilizing a DOX-inducible shIRAK1, similarly resulted in reduced MDSL BM and PB engraftment, reduced splenomegaly, and significantly increased survival (Figures 2.5E-I). Treatment of MDSL-engrafted NSG mice with IRAK-Inh after established disease resulted in an initial MDSL graft reduction (Figure 2.4H), but did not offer any survival benefit. The tool IRAK1 kinase inhibitor used (IRAK-Inh) had not undergone PK/PD evaluation, and thus it’s possible that the IRAK-Inh plasma concentration wasn’t high enough to achieve significant IRAK1 inhibition in vivo. In a pilot experiment BM cells from mice receiving a two-week course of IRAK-Inh (25 mg/kg, I.P., once daily) were examined for pIRAK level via immunoblot. Preliminary results suggested that daily dosing at 25 mg/kg was not sufficient to achieve > 50% on-target inhibition. In light of these data, a twice daily dosing strategy and higher IRAK-Inh concentration would be advisable for future experiments. Nonetheless, our IRAK-Inh tool compound had particularly poor solubility
and thus prevented determination of maximum tolerated dose in mice. Precipitates were evident, even in DMSO, when solution concentrations were > 5 mM. Therefore, it would be desirable to further improve the aqueous solubility of IRAK-Inh through appropriate medicinal chemistry, and determine the PK/PD of IRAK-Inh prior to initiating additional in vivo studies. These recommendations are necessary future steps in determining the pre-clinical efficacy of a small-molecule IRAK1 inhibitor strategy utilizing IRAK-Inh in established MDS.

The experimental evidence provided herein provides compelling proof-of-concept for targeting TLR/IL1R signaling in MDS, and establishes IRAK1 as a rationally derived MDS drug target. IRAK inhibition is an effective therapeutic strategy in MDS cell lines, primary MDS patient cells, and in an MDS xenograft model. The diversity of pre-existing IRAK1 tool compounds should facilitate the rapid synthesis and development of novel lead compounds. Small-molecule inhibitors targeting IRAK1 could represent a new avenue for suppressing dysregulated TLR/IL1R signaling to effectively eliminate MDS-propagating cells.

**Development of novel therapies for AML**

The treatment landscape for AML is similar to that of MDS. The prognosis for AML patients is determined by the underlying biology that drives the leukemic cell. Historically the biology of AML subtypes has been defined by certain chromosomal translocations. However, in recent years, many genetic/epigenetic alterations have been exhaustively catalogued (228, 229), and have improved our understanding of this disease. Perhaps surprisingly, following these exciting discoveries, there has been a lack of clinically meaningful therapeutic developments. Instead, familiar chemotherapeutic strategies that have been employed for several decades continue as front-line therapies. These chemo-strategies can be effective at inducing remission, but ultimately most patients suffer from relapse, usually within months of initial treatment. Therefore, unlike other tumor types, AML has not yet realized the potential value of targeted treatment approaches, and therefore represents a serious unmet need.
Successful development and implementation of targeted therapy for AML will likely result in improved patient outcomes and should be vigorously pursued by the academic and clinical community.

While studying IRAK1 in MDS, we found a similar overexpression in BM cells derived from AML patients (Figure 2.2S). However, in contrast to MDS, pharmacological inhibition of IRAK1 was not effective at slowing the proliferation of primary AML cells, reducing their viability, or decreasing their progenitor activity (Figures 2.3A; 2.3B; 2.3J and 2.3K). Interestingly, some AML cell lines were sensitive to IRAK-Inh (Figures 2.3A-E); perhaps owing to the differential biology exhibited by primary specimens when compared to cell lines. Our integrated gene-expression analysis led to a serendipitous discovery that could provide a potential mechanism of IRAK-Inh resistance. Namely, some cell lines exposed to IRAK-Inh exhibited a compensatory upregulation in BCL2 mRNA and protein levels. For example, the IRAK-Inh-refractory cell line, HL60, exhibited upregulated BCL2 protein levels 24 hrs after exposure to IRAK-Inh (Figure 2.6F). Although upregulated BCL2 expression has been previously observed in cell lines exposed to broadly cytostatic/cytotoxic agents (173), this led us to hypothesize that combining IRAK-Inh with a BCL2 inhibitor would provide an enhanced cytotoxic effect, and induce apoptosis in IRAK-Inh-refractory AML cells. We combined the BH3 mimetic, ABT263, with IRAK-Inh. This resulted in potent, collaborative cytotoxicity in all IRAK-Inh-refractory AML cell lines and primary samples tested (Figure 2.7). Notably, this potent combinatorial effect was observed even in MDS/AML cell lines that didn't exhibit a compensatory increase in BCL2 expression in response to IRAK-Inh. Interestingly this drug combination demonstrated a differential effect on normal CD34+ HSPCs when compared to primary AML and MDS cells. In fact, colony forming cell assays indicated a significant therapeutic window between malignant and healthy cells. Additionally, future efforts should determine if combined BCL2 and IRAK inhibition is a broadly applicable therapeutic strategy in other cancer cell types. Interestingly, one of the general factors differentiating AML from MDS is an increased expression of
prosurvival molecules, like BCL2. Therefore it’s possible that primary AML cells are less sensitive to IRAK-Inh then their MDS counterparts because of higher basal BCL2-family expression.

Mounting evidence suggests that IRAK inhibition synergizes with other relevant targets in AML. Towards this end, Chapter 5 offers a vignette on combined IRAK and FLT3 inhibition as a strategy for mutant-FLT3 AML. Mutations in FLT3 are the most common genetic lesions in AML; approximately one-third of patients harbor an internal tandem duplication (ITD) in FLT3 (FLT3-ITD), while 7% of patients have point mutations at D835 within the activation loop of the kinase (201). Patients harboring FLT3-ITD have particularly poor prognosis as compared to other AML subtypes, whereas the prognostic impact of isolated activation loop mutations is somewhat controversial (201, 230, 231). Not surprisingly the frequency of FLT3 mutations has driven great interest in the development of small-molecule FLT3 inhibitors. First generation FLT3 inhibitors were largely ineffectual and evoked transient marrow responses, owing to poor PK/PD properties (232). More recently, quizartinib (AC220) has emerged as a potent FLT3 inhibitor with significant clinical activity (233, 234). The results of two recent phase II studies of quizartinib demonstrated a composite CR (cCR) rate of 42-47% in the salvage setting (202). Unfortunately most patients achieving a cCR had incomplete hematologic and/or platelet recoveries, and bone marrow responses were short-lived. Thus current clinical evidence suggests that quizartinib may be best used in combination with traditional chemotherapies, or as a bridge to HSCT. One potential reason behind the short-lived marrow response could be due to acquisition of additional mutations in FLT3. In fact, this phenomenon has been documented with the clinical use of quizartinib (235). Additional on-target mutations are typically found at D835 in the activation loop of FLT3, with mutations occurring at the F691 residue at a much lower frequency. These mutations function to render AML cells less sensitive to quizartinib and appear to be associated with disease progression. Interestingly, D835 mutations are not specific to quizartinib, and have been documented with off-label use of sorafenib and sunitinib (236, 237).
As the clinical development of FLT3 inhibitors continues, overcoming the resistance of on-target mutations will be a primary concern.

There are multiple lines of evidence illustrating aberrant NF-κB pathway activation in FLT3 AML (203-208). Work described herein has outlined the activation and overexpression of IRAK1, a mediator of NF-κB activity, in primary AML specimens (Figure 2.S2). Additionally, in vivo synergism between FLT3 and NF-κB pathway inhibitors has been previously demonstrated (238). Thus co-targeting NF-κB signaling along with FLT3 is an attractive rational therapeutic strategy. We therefore hypothesized that IRAK family kinases would be rational co-targets of FLT3. In Chapter 5, we report the structure and cellular activity of four novel imidazo[1,2-a]pyridine tool compounds that function as dual IRAK/FLT3 inhibitors (NCGC00262331, NCGC00241410, NCGC00262376, NCGC00262327) (Figure 5.1). All four compounds have low nM biochemical activity against FLT3; varying activity against IRAK1 and IRAK4, and several related kinases (Table 5.1). In BaF3 cells engineered to express D835Y FLT3, both NCGC00241410 and NCGC00262327 exhibited 4- to 5-fold more potent EC50 values than quizartinib (Table 5.2). Additionally, in a primary human CD34+ AML system, engineered to co-express the MLL-AF9 fusion oncogene and FLT3-ITD (MA9 FLT3-ITD) all four analogues exhibited 14- to 1300-fold more potent EC50 values than quizartinib. In future studies, rescue experiments utilizing the expression of inhibitor-resistant IRAK1 and IRAK4 mutants will be necessary to determine the relative contribution of IRAK signaling towards the cellular potency of this molecule class. Arguably, the human FLT-ITD cell system reported provides a better experimental readout for testing novel anti-FLT3 agents, when compared to BaF3 cells. Although BaF3 are useful in probing requirements for oncogene dependent growth, they are murine pro-B cells, and the underlying genetics may not accurately reflect human AML. Conversely, MA9 FLT3-ITD cells were initially derived from human HSCs, the cell of origin for AML, and thus may more closely recapitulate the biology of this malignancy. When tested in
colony forming assays at a modest dose of 100 nM, only NCGC00262327 was capable of inhibiting the colony formation of human CD34+ MA9 FLT3-ITD cells (Figure 5.2A). Inhibition of colony formation could be rescued by the addition of FLT3L (Figure 5.2B). Thus FLT3 inhibition appears necessary to mediate the cellular effect of this molecule class. Determination of cellular EC\textsubscript{50} values for all analogues in liquid culture with exogenous FLT3L would ultimately validate this finding. Additionally, it’s possible the other analogues could demonstrate activity against colony formation, albeit at a higher dose. Although, the in vivo activity has yet to be demonstrated, the tool compounds reported have markedly better cellular activity than quizartinib in the context of D835 mutant FLT3 and FLT3-ITD AML. Further PK/PD and in vivo characterization is required to determine the suitability of these compounds as potential leads for drug development.

**Mechanisms of IRAK deregulation in hematologic malignancies**

Activation of IRAK signaling can occur through gain-of-function mutations, through loss of a tumor suppressor, and dysregulation of upstream signaling factors. For example, in ABC DLCBL and WM there is a common L265P somatic mutation in MyD88, which drives lymphoma survival through constitutive activation of IRAK4 and IRAK1 (114, 115). Furthermore, the most common cytogenetic aberration in MDS is del(5q); this chromosomal deletion results in the loss of miR-146a expression. Importantly, IRAK1 is a validated targeted of miR-146a, and loss of miR-146a results in IRAK1 derepression (Figure 2.S1) (119-121). However, IRAK1 overexpression and activation occurs in non-del(5q) patients, suggesting additional mechanisms of deregulation. Another example is illustrated in MDS patients that harbor somatic TLR2 mutations; these patients exhibit markedly increased pIRAK4 and pIRAK1 levels, and other MDS patient subsets exhibit overexpression of TLR1, 2 and 6 (122), which could similarly contribute to IRAK1 activation. Moreover, in a related hematologic disease, fanconi anemia, IRAK1 activation similarly coincides with TLR8 overexpression (169). A signaling adaptor
downstream from TLR/IL1R, but proximal to IRAK is IL1RAP. Similar to some TLRs, IL1RAP is overexpressed in high-risk MDS and AML patients (123), and thus may represent another mechanism whereby IRAK signaling is activated in MDS/AML. Another likely possibility is that IRAK transcription is upregulated through epigenetic mechanisms, although this has yet to be demonstrated. Future experiments could delineate an epigenetic effect on IRAK transcription by evaluating loading of DNA-binding factors to IRAK enhancer elements, as well as interrogating the overall chromatin structure surrounding the IRAK gene.

Summary

This thesis has demonstrated the feasibility of targeting TLR/IL1R signaling in the context of MDS and AML. Importantly, this work is the first to uncover IRAK1, a critical TLR/IL1R signaling mediator, as a suitable therapeutic target within this pathway. Through engineering a novel xenograft model of MDS we have provided a platform for testing novel anti-MDS agents. Targeting IRAK signaling, in concert with other relevant survival pathways, provides powerful synergism, as demonstrated with BCL2 and FLT3. Small-molecule inhibitors targeting IRAK family kinases represent an exciting new avenue for drug development.
Bibliography


42. Appelbaum FR. The role of hematopoietic cell transplantation as therapy for myelodysplasia. Best practice & research Clinical haematology. 2011;24(4):541-7.


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