Key signaling pathways in Liquid Cancer : Therapeutic targets, screening tools, and future developments

Addressing leukemia and lymphoma research utilizing biochemical and cell-based assays

Liquid tumors occur in the blood, bone marrow, or lymph nodes and can be classified into three main categories of cancer: leukemia, lymphoma, and myeloma. These types of cancer do not form solid masses, often making diagnosis and treatment different than for solid tumors, such as carcinomas and sarcomas.

Although leukemia and lymphoma are both forms of blood cancer, they affect the body in slightly different ways. The main difference is that leukemia affects the blood and bone marrow, while lymphomas mainly affect the lymph nodes. For both cancers, much progress has been made in the research and development of new treatments, and often these therapies target the signaling pathways that are constitutively activated in specific types of cancer.

In this review we will highlight some of these signaling pathways and illustrate how different assays based on HTRF and AlphaLISA technologies can be utilized in the screening and development of novel therapies for leukemia and lymphoma treatment.

Acute myeloid leukemia

Acute myeloid leukemia (AML) is a fatal hematopoietic malignancy and one of the most common types of leukemia among adults.[1] The condition is characterized by ineffective hematopoiesis (formation of blood cellular components), defects in myeloid differentiation, and an accumulation of myeloid blasts in the patient's bone marrow and peripheral blood. The main treatment modality for patients with AML is chemotherapy using cytotoxic agents. However, around one third of patients fail to show complete response to treatment,[2] and in those older than 60 years of age, only 39% to 64% of patients achieve a complete response.[3]

There are many ongoing approaches for developing innovative and novel therapies for AML, such as antibodies against CD33, epigenetic targets, and T-cell immunotherapy.[3] Gene mutation-targeted drug therapies have also made a breakthrough in recent years, and are showing promising results in patients with AML.[4]

Targeting FLT3 mutations in AML

One of the most common genetic alterations observed in patients with AML are mutations in the gene encoding the FMS-like receptor tyrosine kinase-3 (FLT3).[5] This has led many researchers and pharmaceutical companies to focus their efforts on identifying FLT3 inhibitors as potential therapeutic agents for AML.

FLT3 is a receptor tyrosine kinase (RTK) of the type III RTK family, which plays an important role in the survival and proliferation of hematopoietic cells. FLT3 is activated by the binding of an extracellular ligand (FLT3 ligand) which triggers various downstream signaling pathways, including PI3K/AKT, RAS/MAPK, and STAT5 (Figure 1).

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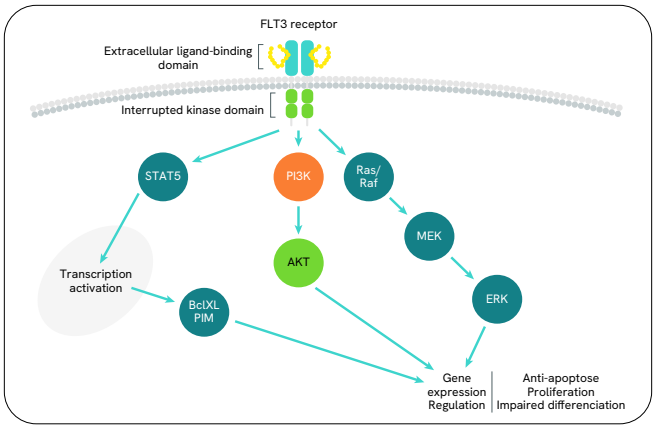


Figure 1: Simplified FLT3 signaling pathways. Ligand binding triggers FLT3 receptor dimerization and phosphorylation leading to activation of downstream signaling cascades including the MAPK3 and PI3K/AKT pathways.

Interestingly, the FLT3 receptor is overexpressed on the majority of AML blasts (70-100%) and mutations of FLT3 are found in approximately one third of AML patients.[3] The majority of FLT3 mutations in AML involve an internal tandem duplication (ITD) in the juxtamembrane domain of the receptor. The second most common mutations are those in the activation loop of the receptor's tyrosine kinase domain. Both mutations cause a conformational change in the receptor and disrupt its autoinhibitory function, thereby resulting in a constitutively active receptor.[6]

Despite significant progress in the development of treatments for patients with FLT3-mutated AML, more potent and selective FLT3 kinase inhibitors are still needed. In 2017, the FDA approved the FLT3 inhibitor midostaurin for the treatment of AML patients with FLT3 mutations, in combination with chemotherapy.[7] Subsequently, the second-generation FLT3 inhibitor, gilteritinib, was approved by the FDA in 2018 as a single agent.[8] Since then, several clinical candidates targeting FLT3 have been reported, including lestaurtinib, tandutinib, sorafenib, KW-2449, and quizartinib.[3] However, the potency and target selectivity of these agents is still under investigation.[3]

Discovery of a novel FLT3 inhibitor – LDD1937

In a recent study,[3] Lee Hyo Jeong *et al.* identified a novel FLT3 inhibitor, LDD1937, through a kinase inhibitory assay of synthesized compounds, which significantly inhibited the growth of AML cells.

These findings built on previous work, where the team reported that indirubin analogues potently inhibited FLT3 kinase.[9] After further development of the indirubin derivatives and their inhibitory activity, the researchers selected the LDD1937 compound for further characterization.

The team first measured the *in vitro* effect of LDD1937 on FLT3 kinase activity, as well as other select kinases (JAK2, JAK3, cMET, and RET), using homogeneous time-resolved fluorescence (HTRF™) KinEASE™ assays (Figure 2). Enzymes were mixed with serially diluted compounds and peptide substrates in a kinase reaction buffer (50 mM HEPES (pH 7.0), 500 µM ATP, 0.1 mM sodium orthovanadate, 5 mM $MgCl_2$, 1 mM DTT, 0.01% bovine serum albumin (BSA), and 0.02% NaN3). After the addition of the reagents for detection, the TR-FRET signal was measured with a VICTOR™ multilabel plate reader. The team also assessed IRAK4 kinase activity using the LANCE™ *Ultra*™ kinase activity assay.

Analysis revealed potent inhibitory activity of LDD1937 against FLT3 kinase, as demonstrated by an IC_{50} of 3 nM (Figure 3). Notably, there was at least a 170-fold difference in the IC_{50} values against FLT3 compared with the other kinases (Table 1).

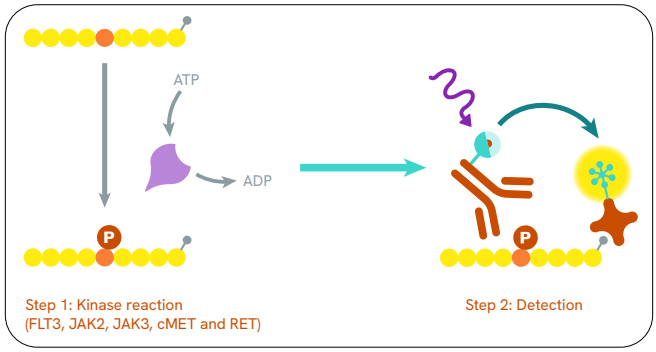


Figure 2: HTRF KinEASE platform assay principle. The kinase is incubated in the presence or absence of compounds and a biotinylated substrate specific for tyrosine kinases. ATP is added to initiate the reaction. The detection step is carried out using a Europium labeled phosphor-specific antibody and Streptavidin (SA) labeled XL665 acceptor. TR-FRET (HTRF) signal generated is proportional to the amount of phosphorylated substrate generated in the reaction.

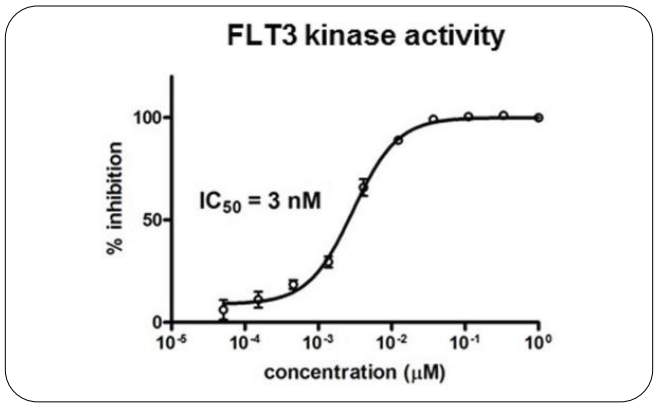


Figure 3: Effect of LDD1937 on the *in vitro* FLT3 kinase activity. Inhibition of kinase activity of recombinant FLT3 was measured with the HTRF assay. Kinase inhibition was calculated with 1% DMSO as a negative control. Data are the mean ± SEM of three independent experiments. Image credit: Lee H, Lee J, Jeong P, Choi J, Baek J, Ahn S *et al.*[3]

Table 1: *In vitro* activity of LDD1937 against select kinases.

Kinase	IC_{50} (μM)
FLT3	0.00300 ± 0.000525
JAK2	0.523 ± 0.0727
JAK3	0.690 ± 0.0599
cMET	0.239 ± 0.0740
IRAK4	0.300 –

Data indicate mean ± S.D.

Further investigation showed that the LDD1937 compound selectively inhibited the growth of MV-4-11 cells (leukemic cells with a FLT3 kinase mutation; GI50 = 1 nM) and induced apoptotic cell death. The team then performed an MV-4-11 xenograft study to examine the efficacy of LDD1937 *in vivo*. The researchers observed a significant reduction in tumor volume and weight compared to the control following intravenous administration of 5 and 10 mg/kg. Writing in Oncotarget, the authors conclude that LDD1937 is a promising therapeutic candidate to treat AML patients because of its ability to suppress tumor cell growth *in vitro* and *in vivo*.

Chronic lymphocytic leukemia

Chronic lymphocytic leukemia (CLL) is a slow, progressive B-cell malignancy characterized by the proliferation and accumulation of B-lymphocytes in the blood, bone marrow, and lymphoid tissues. The condition accounts for around one quarter of new cases of leukemia and is the most common leukemia in adults.[10]

Most patients with CLL are treated with a combination of targeted therapy and chemotherapy. Current targeted therapies include monoclonal antibodies (mAbs), B-cell receptor (BCR) inhibitors, BCL2 inhibitors, Bruton's tyrosine kinase (BTK) inhibitors, and phosphatidylinositol 3-kinase (PI3K) inhibitors. Despite initial response to first-line therapy, CLL is generally incurable and commonly relapses or becomes refractory.[11]

Targeting the PI3K signaling pathway in CLL

The PI3K signaling pathway is one of the most frequently dysregulated pathways in human cancers, including CLL.[12] This pathway is highly conserved, and its activation is tightly controlled by a multistep signaling process (Figure 4)

Activated PI3K phosphorylates PIP2 to PIP3, triggering the activation of protein kinase B (AKT). AKT then mediates numerous downstream cellular functions including angiogenesis, metabolism, growth, proliferation, and survival. Of all classes of PI3K enzymes, the four class I isoforms – PI3Kα, β, δ and γ – have frequently been implicated in cancer.11 The α and β isoforms are expressed ubiquitously, while PI3Kγ is preferentially expressed in leukocytes. Notably, the PI3Kδ isoform is expressed mainly in the cells of hematopoietic lineage, and acts as a second messenger to many cell receptors, including BCRs and chemokine receptors (CXCR4 and CXCR5).[11]

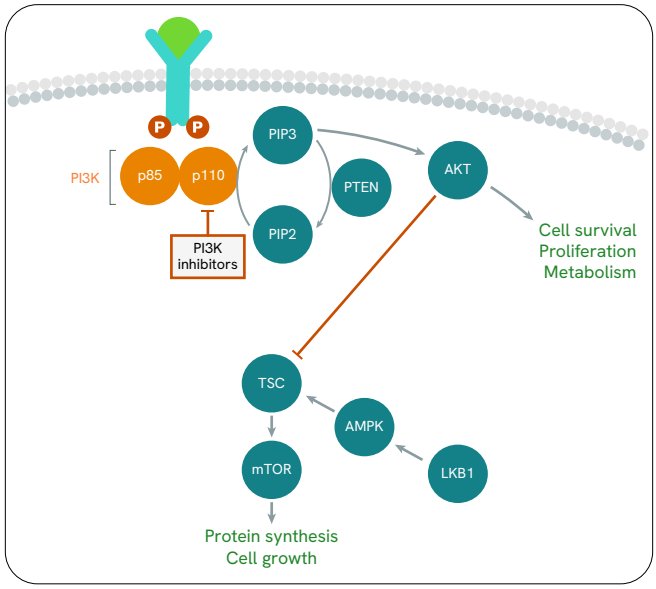


Figure 4: Simplified PI3K/AKT signaling pathway. Binding of external stimuli via various cellular receptors triggers the intracellular interaction of PI3K p85 subunit with the activated receptor, while the p110 subunit initiates the conversion of PIP2 to PIP3. PIP3 stimulates the activation of AKT1, which results in the downstream signaling cascade leading to increased cell growth and proliferation.

Inhibition of the PI3K pathway is considered an attractive therapeutic approach for treating CLL. Indeed, three PI3K inhibitors have already been approved by the FDA for the treatment of CLL: idelalisib (targeting PI3Kδ), duvelisib (targeting PI3Kδ and γ), and copanlisib (targeting PI3Kα and δ).[13] However, serious adverse drug reactions can be difficult to avoid, and novel PI3K inhibitors with improved safety and long-term maintenance are still needed.

A novel selective inhibitor of PI3Kδ

To address this unmet need, a team of researchers led by Lei Fan and Jianyong Li, from the Jiangsu Province Hospital, Collaborative Innovation Center for Cancer Personalized Medicine in Nanjing, China, used biochemical and cell-based assays to evaluate the *in vitro* antitumor activity of SHC014748M – an oral selective inhibitor of PI3Kδ.11 First, to determine the selective inhibition of SHC014748M, evaluation against a panel of 50 different kinases closely related to BCR signal were conducted. Kinases were seeded in 384-well plates and treated with 10 different concentrations of each compound (ranging between 0.05 nM and 10 µM) in duplicate. Fluorescence polarization was read using a 2104 EnVision™ Multilabel Plate Reader.

Results revealed that SHC014748M inhibited the activity of all four Class I PI3K isoforms, as demonstrated by IC_{50}s of 0.77 (PI3Kδ), 236 (PI3Kα), 96 (PI3Kβ), and 101 nM (PI3K γ). Of note, SHC014748M was 125- to 306-fold more selective for PI3Kδ inhibition compared with the three other Class I PI3K isoforms.

To further investigate the cellular selectivity of SHC014748M against these isoforms, inhibition of AKT phosphorylation was measured using AlphaLISA™ technology (Figure 5). RAJI (PI3Kδ), PC-3 (PI3Kβ), RAW264.7 (PI3Kβ), and C2C12 (PI3Kα) cells were cultured with various concentrations of SHC014748M or idelalisib for 30 minutes, then stimulated for 10 minutes by anti-IgM, LPA, C5a, or IGF-1, respectively. pAKT was then measured by the AlphaLISA™ *SureFire® Ultra™* phospho-AKT1 (Ser473) Kit and/or the AlphaLISA *SureFire Ultra* total AKT1 Kit on a 2104 EnVision™ Multilabel Plate Reader (Figure 5A).

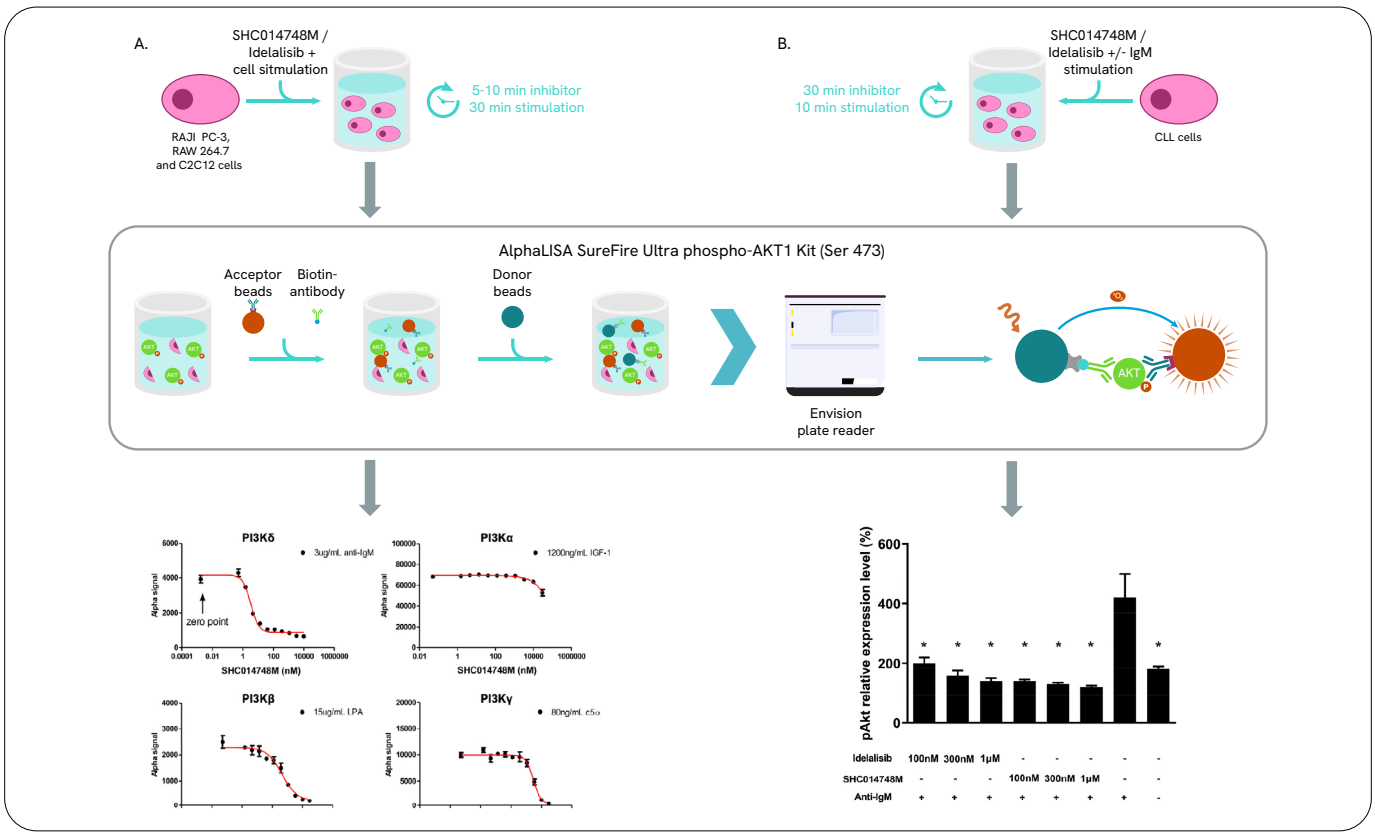


Figure 5: Assessment of PI3K downstream AKT1 signaling in cultured cell lines and primary CLL cells using AlphaLISA *SureFire Ultra*. Cultured cell lines were incubated with SHC014748M followed by stimulation of anti-IgM (Raj), IGF-1(C2C12), LPA (PC-3) or C5a (RAW 264.7) (A). Primary CLL cells were incubated with idelalisib or SHC014748M followed by stimulation using IgM (B). After cell treatments, cell lysates were incubated with antibodies specific for phosphorylated AKT Ser473 conjugated to AlphaLISA detection reagents. For CLL primary cell experiments (B), total AKT1 was also measured and the resulting data is representative of the percent of phosphorylated AKT measured compared to the total AKT measured in the cell samples.

Analysis revealed that SHC014748M potently inhibited phosphorylation of AKT, specifically downstream of PI3Kδ on threonine-308 or serine-473 (IC_{50} = 3.32 nM). However, no significant inhibition was observed against AKT phosphorylation in the other Class I PI3Ks. By contrast, idelalisib also potently inhibited pAKT of PI3Kδ (IC_{50} = 5.55 nM), but its selectivity to this isoform was much lower than SHC014748M.

The researchers then used AlphaLISA to evaluate the pAKT levels in primary leukemia cells derived from CLL patients in the presence or absence of various concentrations of SHC014748M or idelalisib (Figure 5B). They report that SHC014748M efficiently decreased the levels of pAKT to unstimulated levels at the concentration of ≥100 nM, whereas idelalisib could only achieve this at a concentration of ≥300 nM. These results suggest that SHC014748M had antitumor efficacy equivalent to or better than that of idelalisib, and the team suggests SHC014748M could be a promising novel compound in the treatment of CLL.

Tumor suppressor p53 signaling pathway

The tumor protein p53 signaling pathway is a particularly attractive target for the development of anti-cancer therapies. Specifically, p53, also known as the "guardian of the genome", has many roles as a tumor suppressor, including its ability to induce cell cycle arrest, DNA repair, senescence, and apoptosis. Activation of p53 can occur in response to a number of cellular stresses, such as DNA damage or metabolic stress, leading to the activation of various downstream target genes. Of note, the p53 gene is the most frequently mutated gene (>50%) in human cancer, which suggests that it plays a crucial role in preventing cancer formation.

p53 activity is mainly regulated by the homologous p53-binding proteins, Mdm2 and MdmX. Under normal or non-stressed conditions, Mdm2 inhibits p53, preventing unwanted cell cycle arrest or even cell death. However, in response to cellular stress Mdm2 is post-translationally modified and temporarily stops its inhibition of p53 (Figure 6). Mdm2 inhibits p53 activity in two ways: firstly, by binding to the transactivation domain of p53, thereby inhibiting its ability to cause transcription of its targets; and secondly, by acting as an E3 ubiquitin ligase of p53.[14]

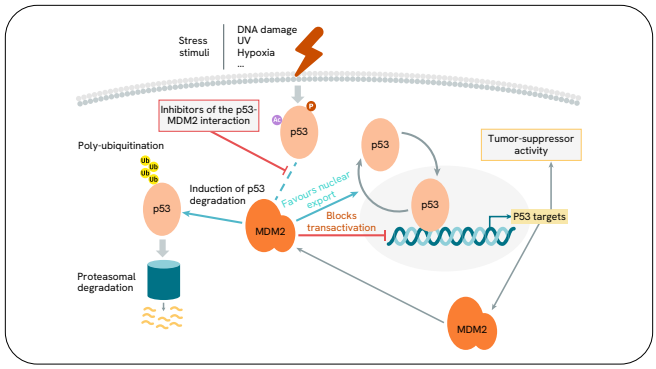


Figure 6: Interaction of p53 and Mdm2. Stress stimuli leads to increased expression and activation of p53 protein which triggers downstream signaling events leading to tumor suppressor activity (apoptosis and cell cycle arrest). p53 also triggers the expression of MDM2 which results in an autoregulatory feedback loop as Mdm2 induces p53 degradation via the proteasome, favoring nuclear export of p53 and downregulation of tumor suppressor activity.

Mdm2 and MdmX both contain a conserved C-terminus really interesting new gene (RING) domain. Studies have shown that the MdmX-Mdm2 RING-RING interaction is essential for p53 polyubiquitination and proteasome-dependent degradation,[15] while disruption of the Mdm2/MdmX complex results in p53 activation.[14] These findings, along with the knowledge that p53 is rarely mutated in leukemia or lymphoma,[15] suggest that the MdmX-Mdm2 RING-RING interaction could be a potential interface for drug targeting, and that screening for E3 ligase inhibitors could help in the development of anti-cancer therapies.

Targeting the RING domains of Mdm2-MdmX E3 complex

With this in mind, W Wu, C Xu, X Ling *et al.* set out to identify small molecule inhibitors for the E3 ligase activity of the Mdm2-MdmX E3 complex using a time-resolved fluorescence resonance energy transfer (TR-FRET)-based E3 activity assay.[15] This system uses homogeneous time-resolved fluorescence (HTRF) to quantify ubiquitin chain reactions.

As demonstrated in Figure 7, two fluorophores that generate TR-FRET were conjugated to ubiquitin (Ub-K, ubiquitin cryptate) and anti-HA antibody (anti-HA-XL665). Once the ubiquitin chains are assembled on HA-Mdm2 and MdmX proteins, these two fluorophores will be brought in proximity for TR-FRET to occur. The HTRF emission was measured at two different wavelengths: 615 nm (donor) and 665 nm (acceptor).

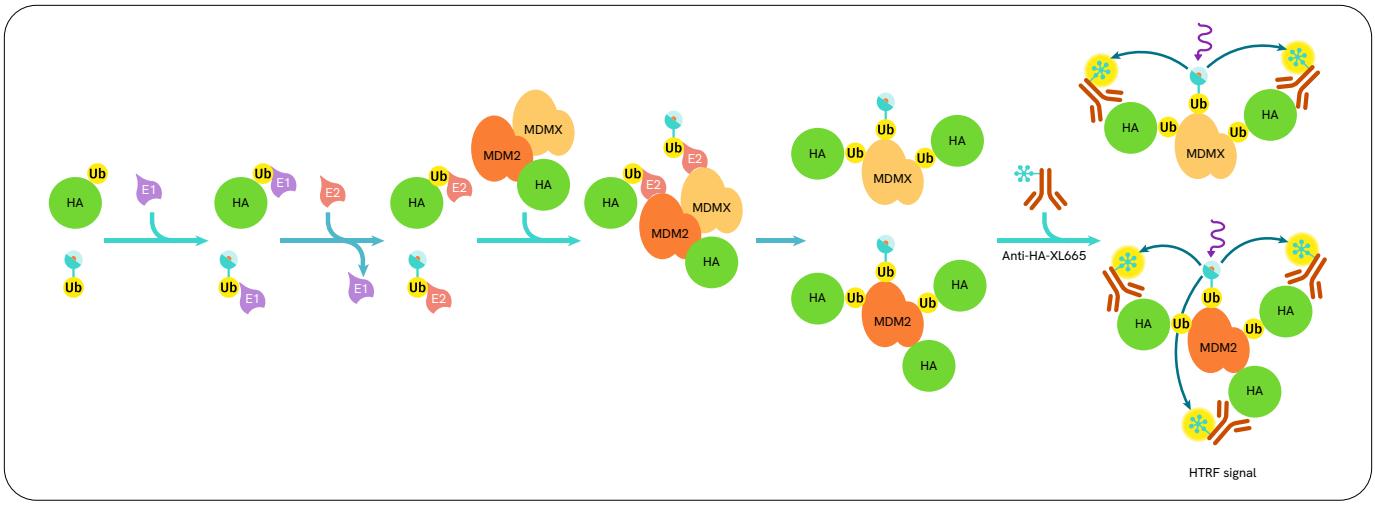
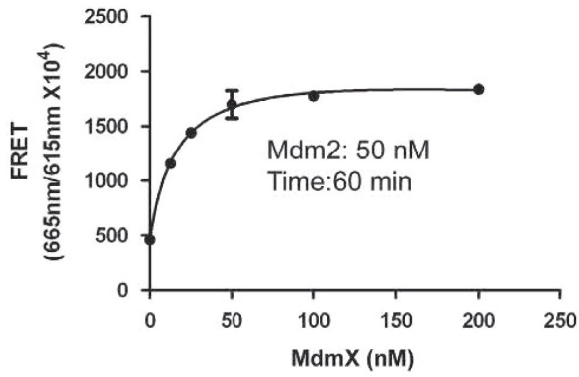
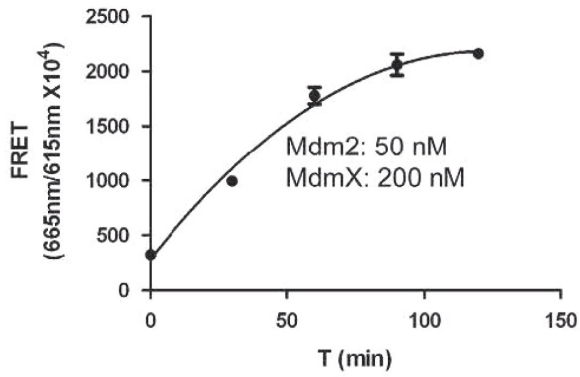
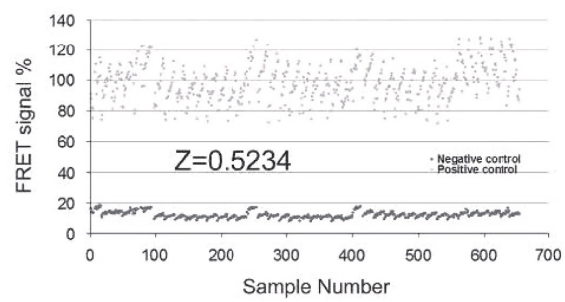


Figure 7: Mdm2-MdmX ubiquitation HTRF assay principle. Cryptate labeled ubiquitin was pre-incubated with HA labeled ubiquitin followed by the initiation of the ubiquitation reaction by adding pre-mixed E1/E2 enzymes and MdmX and HA-tagged Mdm2 in reaction buffer. After 90 minutes, the reaction was stopped with the addition of detection buffer containing anti-HA XL665. Generation of TR-FRET (HTRF) signal occurs when the ubiquitin labeled cryptate comes into close proximity of the anti-HA XL665 antibody with the amount of HTRF signal generated being proportional to the amount of ubiquitin chains assembled.

The team reports that addition of MdmX produced an approximately eight-fold increase in TR-FRET signals in an MdmX concentration-dependent and reaction time-dependent manner (Figure 8A and 8B, respectively). When they performed an initial screen of around 650 samples, the Z'-factor of their HTS assay was determined to be 0.52 (Figure 8C), indicating that it was a suitable and reliable HTS screening assay. A further screen of 55,230 compounds identified 119 hits at 90% inhibition cutoff and 371 hits at 70% inhibition cutoff (Figure 8D).







	98% inhibition	90% inhibition	70% inhibition
Positive hits	13	119	371
Positive rates	$2.3x10^{-4}$	$2.2x10^{-3}$	$6.7x10^{-3}$

Figure 8: Small molecule inhibitor screening of the Mdm2-MdmX interaction using HTRF. Optimization of MdmX protein concentration using a fixed concentration of Mdm2 and a fixed reaction time (A) and optimization of reaction time (B) using fixed concentrations of Mdm2 and MdmX using the HTRF assay principle shown in Figure 7. (C) HTS screen performed using optimal HTRF reaction conditions with Z' determination. (D) Summary of hit rate using different HTRF inhibition cutoff thresholds using Chembridge DIVERset library.

Among seven specific MMRis (Mdm2–MdmX RING domain inhibitors) identified, the team further analyzed MMRi64 in vitro. Their study revealed that MMRi64 disrupts the Mdm2-MdmX interaction and inhibits the E3 ligase activity of Mdm2–MdmX, without affecting the E3 ligase activity of Mdm2 RING domain homodimers. Furthermore, MMRi64 was shown to induce PUMA (p53 upregulated modulator of apoptosis) but strongly downregulate MdmX and Mdm2, consequently activating the apoptotic arm of the p53 pathway in leukemia/lymphoma cells without the induction of growth arrest.

The authors conclude that future studies will be needed to address the biophysical property of drug-target interaction, p53/Mdm2/MdmX-dependency, and the issues of off-targets and genotoxicity in the lead optimization process

Conclusion

As discussed in this review, there are several signaling pathways that have been implicated in the development of liquid cancers. Alterations in these pathways can prevent normal cellular functions, such as controlled cell proliferation, growth, and survival, ultimately leading to cancer progression. Many of the proteins currently under investigation as possible targets for cancer therapy are proteins that are components of these signaling pathways. Here, we provided an overview of several important pathways implicated in leukemia and lymphoma, and how researchers are utilizing novel assay and screening approaches to help develop novel therapies targeting these pathways.

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Revvity, Inc.
940 Winter Street
Waltham, MA 02451 USA
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