

**Using Small Molecules to Inhibit an E2A-PBX1:CBP Interaction
Involved in Acute Lymphoblastic Leukemia**

by

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Abstract

E2A-PBX1 is expressed as a consequence of a recurring chromosomal translocation seen in 5% of acute lymphoblastic leukemia cases. We recently reported that substitution of a leucine residue (L²⁰A) within the N-terminal transcriptional activation domain (AD1) of E2A-PBX1 markedly impairs binding to the KIX domain of CBP/p300 and, importantly, leukemia induction in a mouse bone marrow transplantation model. Since both the protein-protein interaction and consequent leukemogenesis rely on a focal contact point and might therefore be susceptible to antagonism by small molecules, we devised a cell-free assay based on fluorescence anisotropy (FA) to detect binding of a fluorescently labeled peptide derived from AD1 of E2A-PBX1 (FITC-E2A) with recombinantly expressed KIX domain. The optimized FA assay reveals a dissociation constant of 2 μ M for the wild-type interaction and correctly detects disruption of the complex by naphthol AS-E phosphate, a compound previously shown to antagonize KIX binding. The optimized FA assay was used to screen the Prestwick, Spectrum and Chembridge libraries containing 12400 compounds in total. Of the initial 43 positive hits from the libraries, 10 caused a reproducible decrease in FA. Since intrinsic small molecule fluorescence can produce false positive results in the FA-based screen, intrinsically fluorescent compounds were excluded from further analysis unless they could be shown to bind to KIX. Two hits, L1 and C2, were intrinsically fluorescent but demonstrated KIX interactions and one hit, P9, was not intrinsically fluorescent. These three compounds were tested for their ability to inhibit binding of a larger portion of E2A (residues 1 to 483) to full length CBP in a pull down assay with only compound P9 demonstrating efficacy. Further characterization of P9 by NMR showed no binding to KIX, however evaluation by FA showed binding to FITC-E2A with a 20 μ M affinity. A cell-based cytotoxicity assay demonstrated that compound P9 was slightly more toxic on leukemic cells that express E2A-PBX1, compared to leukemic cells lacking E2A-PBX1 expression. Mammalian

two-hybrid analysis did not provide details of the effects of P9 on the E2A:KIX interaction. We expect the identification of a novel compound, P9, capable of disrupting the oncogenic E2A-PBX1:CBP interaction, to guide the development of effective, less toxic leukemia drugs and provide new tools for elucidating the molecular mechanisms of leukemia induction by E2A-PBX1.

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List of Abbreviations

aa	amino acid
AD	activation domain
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
AT	acetyltransferase
BCR	B cell receptor
BD	bromodomain
bHLH	basic helix-loop-helix
β -Me	beta mercaptoethanol
CBP	CREB binding protein
CH	cysteine/histidine rich
CREB	cyclic AMP response element binding
EBF	early B cell factor
EPM	E2A-PBX1-immortalized myeloid
FA	fluorescence anisotropy
FITC-E2A	carboxyfluorescein-labeled E2A
FITC	fluorescein
FBS	fetal bovine serum
GCs	glucocorticoids
GM-CSF	granulocyte macrophage-colony stimulating factor
GST	glutathione-S-transferase
HAT	histone acetyltransferase
HCM	homeodomain
HOX	homeobox
Ig	immunoglobulin
IgH	immunoglobulin heavy
IPTG	isopropyl β -D-1-thiogalactopyranoside
ITC	isothermal titration calorimetry
K_D	Dissociation constant
KIX	CREB binding domain
L ²⁰ A	point mutation leucine to alanine at residue 20

LXXLL	Leucine-X-X-Leucine-Leucine
MAD	mean absolute deviation
MES	2-(N-morpholino)ethanesulfonic acid
MLL	mixed lineage leukemia
NaP	naphthol phosphate
NK	natural killer
NLS	nuclear localization signal
NMR	nuclear magnetic resonance
PBX1	pre-B cell leukemia homeobox 1

Chapter 1

Introduction

1.1 General overview

In a subset of acute lymphoblastic leukemia (ALL) cases, chromosomal translocation 1;19 targets the *E2A* and *PBX1* genes and results in the expression of a chimeric fusion protein, E2A-PBX1. The oncogenic potential of E2A-PBX1 has been demonstrated in several experimental models, caused in part by an interaction with the transcriptional co-activator CREB binding protein (CBP)¹. This interaction is localized to an amino-terminal activation domain (AD1) of E2A-PBX1 and the KIX domain of CBP. This thesis describes high-throughput screening for small molecule compounds that disrupt the E2A-KIX interaction. Ultimately, a single compound, called P9, is identified that disrupts the interaction, apparently by binding directly to the amino-terminus of E2A. This compound demonstrates cytotoxicity in leukemic cells that express E2A-PBX1, and can be used as a tool for investigating the biological effects of E2A-PBX1 in leukemic cells. It also serves as a novel lead compound for the development of a more specific and less toxic therapy for this subset of ALL cases.

1.2 Acute lymphoblastic leukemia

ALL remains the most prevalent form of cancer amongst children and young adults². Approximately 4500 Canadians were diagnosed with leukemia in 2008, with children and adolescents accounting for one third of these cases³. Although the peak incidence of ALL is in children between the ages of 2 and 5 years, it also occurs in adults and the response to treatment in this age group is far inferior⁴. ALL is characterized by the rapid accumulation of B or T cell progenitors in the bone marrow and circulating blood. It is associated with a block in lymphoid differentiation at any stage of development, from hematopoietic stem cells to mature circulating B

or T cells². The immunophenotypes of leukemic blasts indicates that in most cases the disease originates from B-lymphoid progenitor cells (85%), while the remaining 15% of cases have a T-lineage origin⁵.

ALL is heterogeneous in that many different types of genetic lesions may contribute to the neoplastic transformation of normal hematopoietic progenitors into neoplastic cells, resulting in the deregulation of normal differentiation, proliferation and survival⁶. Non-random somatic chromosomal translocations are strikingly common in ALL, where they are found in up to 65% of cases by karyotype analysis⁷. Chromosomal translocations may activate oncogenes by juxtaposing nucleotide sequences that normally reside on separate chromosomes. This results either in the production of a structurally abnormal “fusion protein” or excessive production of a structurally normal protein. Translocations associated with ALL often target transcription factors, which interact with DNA to control target gene transcription. The expression of transcription factors is a cell-specific, highly regulated and temporally coordinated process⁸. Transcription factors regulate the differentiation of many cell types; they allow for the maturation of hematopoietic cells from pluripotential stem cells to mature circulating blood cells⁷.

Translocations which result in the in-frame fusion of two transcription factors can lead to the expression of a chimeric transcription factor containing domains derived from each of the targeted parent proteins. In many cases, the fusion proteins retain DNA binding ability from one transcription factor and regulatory domains from the other. Several translocations are especially prevalent in ALL: TEL-AML1 t(12;21), E2A-PBX1 t(1;19), BCR-ABL t(9;22), and various rearrangements of the *MLL* gene⁹. Each translocation results in a specific gene expression pattern of differentially regulated downstream genes, defining subtypes of ALL¹⁰. Although there is a role for chimeric fusion proteins in the progression of ALL, there is also a requirement for additional genetic events to acquire full transformation of lymphoblasts¹¹.

Current treatment for ALL consists of various types of chemotherapeutic agents administered in three phases; remission-induction, intensification, and consolidation therapy⁹. Due to the vast improvement of treatment measures over the last few decades, these treatments are curative in up to 80% of childhood cases and 40% of adult cases; however they are extremely toxic. Remission-induction phase includes treatment with various chemotherapeutic drugs, including glucocorticoids (GCs), vincristine, asparaginase and anthracyclines. GCs act on the cytosolic glucocorticoid receptor and result in the differential regulation of many different target genes¹². They have particular effects in the immune system, where they are implicated in cell cycle arrest and consequent apoptosis of lymphoblasts¹³. Although credited with successful remission induction of many patients, GCs are associated with serious adverse effects that include obesity, hypertension, diabetes mellitus and immunosuppression¹⁴. In the past, t(1;19) ALL has been associated with poor responsiveness to anti-metabolite-based treatments, and these patients have required more intense chemotherapeutic regimens⁹. Intensification involves increased doses of methotrexate and asparaginase, which can result in serious side effects such as pancreatitis and thrombotic complications, sometimes leading to death¹⁵. Current ALL research focuses on tailoring specific treatments for ALL patients based on their genetic subtypes in order to reduce toxic chemotherapeutic side effects. Targeted treatment of ALL, such as the use of the tyrosine kinase inhibitor imatinib mesylate for BCR-ABL positive ALL cases, has demonstrated positive effects. Imatinib treatment has increased complete remission and disease-free survival, and improved the quality of life of these patients^{4, 16}.

Of particular interest in our lab is the second most common non-random chromosomal translocation in ALL, t(1;19), which targets the *E2A* gene and the *PBX1* gene. This translocation is found in approximately 5% of ALL cases. The majority of patients with the t(1;19) translocation express the chimeric protein E2A-PBX1, which results in a block in differentiation

of B-lineage cells. Immunophenotyping blasts of these ALL patients has shown that many of their cells are blocked at the pre-B cell stage of development².

1.2.1 Normal B-lymphopoiesis

Like all blood cells, B cells are derived from hematopoietic stem cells in the bone marrow after birth. The common lymphoid progenitor can differentiate into B, T, or natural killer (NK) cells¹⁷. B cells then differentiate into pro-B cells, in which little or no immunoglobulin (Ig) chain rearrangements are detectable. Progression into the pre-B stage of development involves *Ig* gene rearrangements and cytoplasmic expression of the pre-B cell receptor, composed of mu heavy chain, the product of a successfully rearranged *IGH* gene, bound to surrogate light chains and cytoplasmic proteins involved in signal transduction¹⁸. Expression of the pre-BCR on the cell surface and successful receptor signaling leads to rapid cellular proliferation, the commencement of light chain rearrangements and subsequent differentiation of pre-B cells into immature B cells. Various selection events in the bone marrow and spleen by successful signaling through the B cell receptor (BCR) then dictate the successful development of immature B cells into mature circulating B cells, available for the immune response¹⁹.

1.3 E2A proteins

The *E2A* gene on chromosome 19, also called *TCF3*, codes for two proteins, E12 and E47, which result from differential splicing of a single pre-mRNA exon. The two E2A proteins share 80% sequence identity with the differences mainly occurring in their C-terminal basic helix-loop-helix (bHLH) domain (**Figure 1-1 A**)²⁰. E2A proteins are founding members of the class I bHLH family of transcription factors. Along with HEB and E2-2, E2A proteins are also referred to as “E-proteins”²¹. The HLH portion of the bHLH domain is formed by two amphipathic helices joined by a flexible loop. This domain mediates homo- and

heterodimerization of these proteins. Directly N-terminal to the HLH portion is a stretch of basic residues which, upon dimerization, interacts with the major groove of DNA at “E-boxes” with the nucleotide sequence CANNTG²². E2A homodimers interact with E-boxes contained within the Immunoglobulin Heavy (IgH) enhancer, which results in the initiation of B cell-specific *IGH* gene expression and rearrangement (**Figure 1-1 B**)²³. Many B cell-specific genes are controlled by E2A proteins, and are involved in the initiation and progression of B cell development and differentiation. bHLH proteins have important roles in the regulation of growth and development in many tissue types, including hematopoietic, neural, muscular and dermal cells²⁴. E2A proteins are involved in cell-type specific differentiation through the formation of heterodimers with tissue-specific class II proteins. These heterodimers are mainly in non-lymphoid tissues, and are involved in controlling events such as skeletal muscle differentiation or pancreatic regulation²⁵.

1.3.1 Transcriptional activation domains of E2A proteins

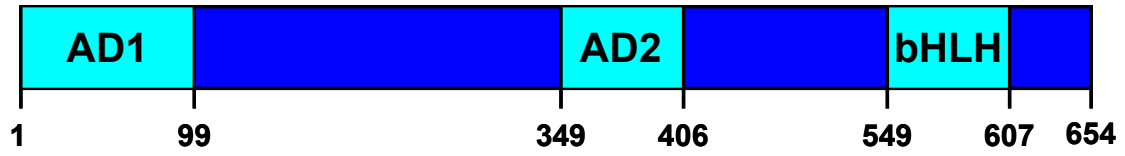
It has long been known that the role of E2A proteins as transactivators of gene transcription has been linked to the N-terminal portion of these proteins²⁶. The fusion of various segments of E2A to GAL4 DNA-binding domains demonstrated the importance of two distinct N-terminal domains in the transactivational abilities of E2A. These two domains were able to transactivate reporter genes that rely on GAL4 binding domains near their promoters for expression²⁷. The activation domains are called AD1 at the extreme N terminus (aa 1-99), and AD2 found in the central region of the protein (aa 349-406) (**Figure 1-1 A**)²⁷. AD1 and AD2 are highly conserved in E proteins, indicating an important role for these domains²⁸.

Transcriptional activators such as E2A proteins are often part of multi-protein complexes, in which transcriptional coactivators mediate interactions between transcriptional machinery, chromatin remodeling proteins, and DNA binding transcription factors (**Figure 1-1 B**)²⁹. Proteins that have a role in chromatin remodeling are important in DNA transcription, allowing for

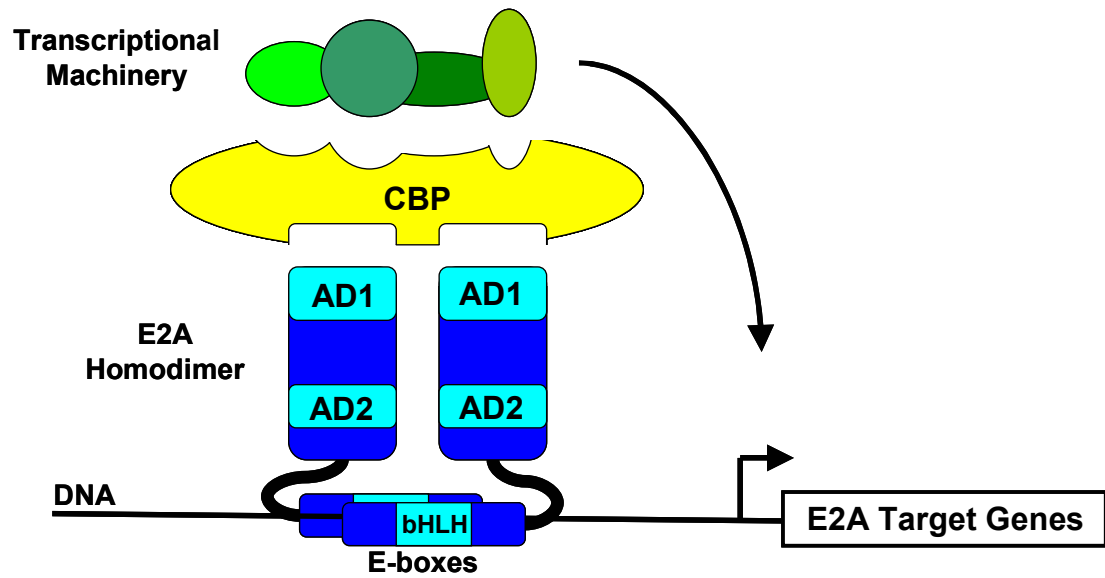
Figure 1-1 Domain structure and functional model of E2A proteins

- (A) Domain structure of the E2A gene products, E12 and E47. E2A proteins have two transcriptional activation domains: AD1 at the extreme N-terminus (residues 1-99) and AD2 located more centrally (residues 349-406). The bHLH domain located near the C-terminal end of the protein mediates homo- or heterodimerization and DNA binding to enhancer elements that contain E-boxes.
- (B) A model of E2A function and target gene regulation. E47 forms homodimers in B cells that recognize E-boxes located within enhancer elements of target genes. E47 homodimers recruit transcriptional co-activators which recognize protein-protein interaction domains within AD1 and AD2 of the E2A proteins. The transcriptional co-activators (such as CREB binding protein, CBP) catalyze acetylation or other post-translational modifications of histones and other chromatin-associated macromolecules and bridge interactions between the DNA-bound E2A proteins and transcriptional machinery proteins resulting in transcriptional induction of E2A target genes.

A E2A proteins (E12 and E47)



B



transcription factors to access DNA by repositioning nucleosomes³⁰. Often co-activators are the center of these large multi-protein complexes, and contain acetyltransferase (AT) catalytic domains which mediate the transfer of an acetyl group to histone proteins to, in turn, promote gene transcription²⁹. Our lab has shown that a highly conserved motif within the helical portion of AD1, LXXLL (aa 16-20), mediates interactions with transcriptional coactivators³¹. LXXLL motifs mediate a number of protein-protein interactions involved in transcriptional regulation. They are highly conserved amongst proteins which interact with the transcriptional coactivator CBP³².

1.3.2 E2A proteins are regulators of B cell development

E2A proteins are the major regulators of lymphoid development, with important roles at multiple stages of B cell differentiation. This was primarily evidenced by the fact that although E2A-null (E2A^{-/-}) mice appear relatively normal, they exhibit a complete block in B cell differentiation at the pro-B stage, before *Ig* gene rearrangements, and often develop T-cell lymphomas²⁰. Enforced expression of E12 or E47 partially restores the normal phenotype in mice, and expression of both E2A proteins completely restores it^{33,34}. E2A proteins are expressed relatively abundantly in lymphoid cells at many stages of development and differentiation, with important roles in B lineage determination, pre-B cell differentiation and class type switching during the immune response³⁵. At the first stages of B cell development, E2A proteins induce the expression of additional transcription factors, including early B cell factor (EBF) and Pax5, that promote B lineage determination and suppress differentiation into other lineages³⁶. E2A proteins are required for the survival of pro-B cells and for the transition from pro-B to pre-B cells³⁵. These various roles for E2A proteins at multiple stages of B cell lymphopoiesis indicate a crucial role for these proteins in B cell ontogeny and function.

1.4 PBX1 proteins

The *PBX1* (pre-B cell leukemia homeobox 1) gene on chromosome 1 also encodes a DNA binding transcription factor. PBX1 has a C-terminal DNA binding homeodomain (HD), which is formed by three alpha helices with a central hydrophobic core (**Figure 1-2 A**)²⁰. PBX1 was initially discovered as the protein fused to the N-terminal portion of E2A in t(1;19) ALL cases. PBX1 has two isoforms, PBX1a and PBX1b, which are generated by alternate splicing of pre-mRNA and differ at the C-terminus³⁷. PBX1a is a longer isoform with 430 aa, whereas PBX1b is the shorter variant, comprising 347 aa. HD proteins are a large family of DNA binding proteins involved in the transcriptional regulation of genes controlling embryonic development and tissue homeostasis³⁸. Homeobox (HOX) proteins are also HD proteins, which control cell fate and segmental patterning. The affinity and specificity of PBX and HOX interactions with DNA are greatly increased upon forming HOX:PBX1 heterodimers, which then target HOX/PBX1 target genes (**Figure 1-2 B**). This interaction is mediated by the HOX cooperativity motif (HCM) within PBX1.

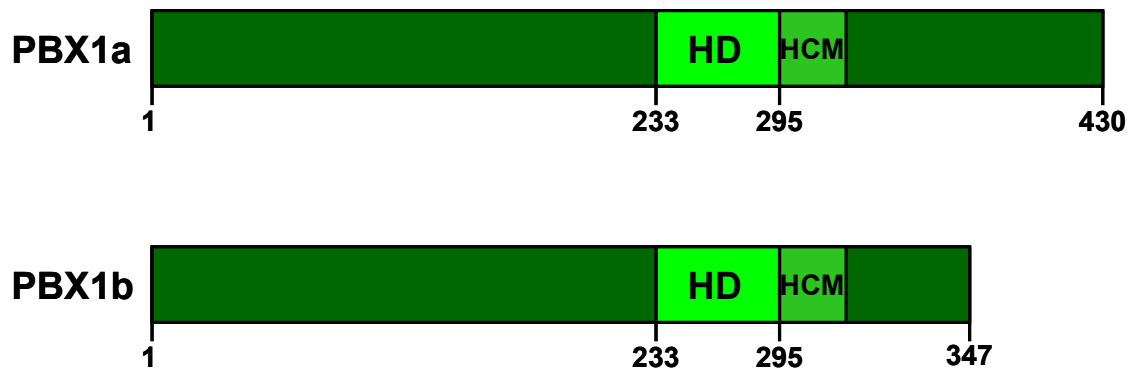
1.4.1 The role of PBX1 in development and hematopoiesis

Expressed widely in many tissues, PBX1 is thought to have roles in embryogenesis, cell fate determination, and tissue homeostasis²⁰. PBX1 null (-/-) mice die relatively early in embryogenesis due to severe developmental defects. These include severe hypoplasia of several internal organs including lungs, liver, and skeleton, or aplasia of the spleen³⁹. In addition to having a crucial role in embryonic development generally, PBX1 has been implicated in hematopoiesis. PBX1 expression can be detected in hematopoietic progenitor cells during embryonic development; PBX1b and PBX3a are expressed in the earliest stages of hematopoiesis⁴⁰. PBX1 (-/-) mice experience multiple hematopoietic defects that include anemia, reduction in the pluripotentiality of hematopoietic stem cells, and depletion of common myeloid

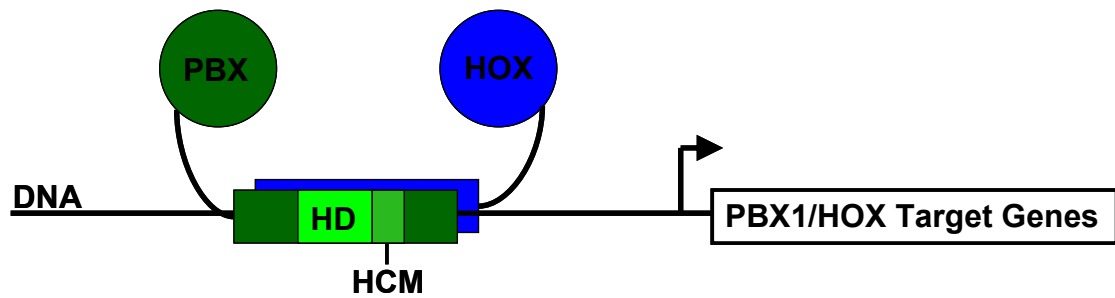
Figure 1-2 Domain structure and functional model of PBX1 proteins

- (A) Domain structure of the *PBX1* gene products, PBX1a and PBX1b. PBX1 proteins contain a DNA binding HD at the C-terminal end of the protein, which mediates interactions with DNA. The HCM, found directly C-terminal to the HD, is involved in HOX interactions.
- (B) A model of PBX1 function and target gene activation. PBX1 interacts with DNA subsequent to forming heterodimers with HOX proteins; this interaction is mediated by the HD of PBX1 and a short run of amino acids N-terminal to the HD in the HOX partner. The interaction also involves the HCM within PBX1. Heterodimerization and DNA binding permits transcriptional regulation of PBX1/HOX target genes, potentially involving the recruitment of co-regulator proteins.

A PBX1 proteins (PBX1a and PBX1b)



B



progenitors⁴⁰. PBX1 is not required for the maintenance of mature B cells, or in the differentiation at later stages of development beyond the pro-B cell stage. However, it has been implicated in the transition from hematopoietic stem cells to committed B cells, acting as one of the earliest transcription factors in B cell development⁴¹.

1.5 E2A-PBX1 chimeric protein

The second most common non-random chromosomal translocation in ALL, t(1;19)(q23;p13.3) targets the *E2A* gene and the *PBX1* gene, which results in the expression of the chimeric protein E2A-PBX1. The *E2A-PBX1* gene on chromosome 19 encodes for the amino terminal two thirds of E2A (aa 1-483) and the majority of PBX1 (aa 89-430 for PBX1a and aa 89-347 for PBX1b) (**Figure 1-3 A**). No major differences have been found in the functions of E2A-PBX1a and E2A-PBX1b, therefore collectively they will be referred to as E2A-PBX1. E2A-PBX1 includes the HD of PBX1, thus preserving DNA binding ability. However, unlike E2A proteins, which bind to E-boxes on DNA, E2A-PBX1 interacts with PBX1-responsive gene elements as directed by the PBX1 HD (**Figure 1-3 B**). The portion of E2A included in E2A-PBX1 is identical in both E12 and E47, and retains the two activation domains, AD1 and AD2.

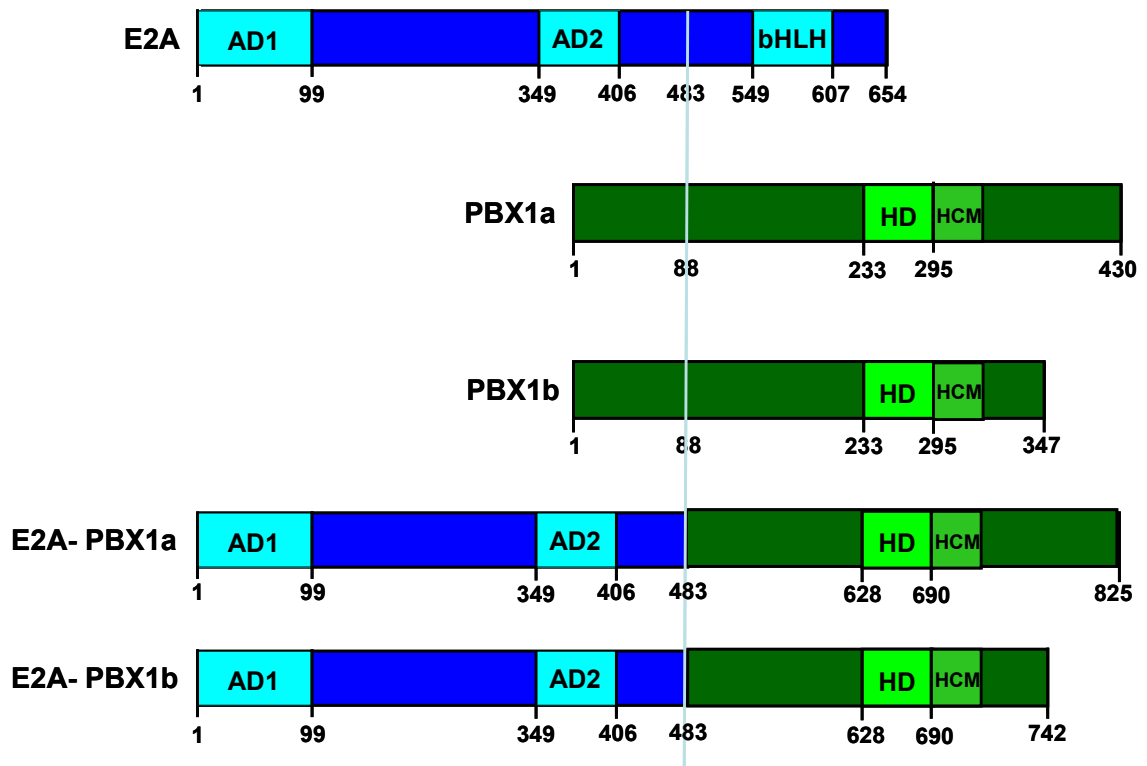
1.5.1 Oncogenic potential of E2A-PBX1

The oncogenic transformation potential of E2A-PBX1 has been demonstrated using a number of experimental models. Insertion of E2A-PBX1 cDNA into a retroviral vector and subsequent expression in fibroblasts results in the formation of transformed foci and anchorage-independent growth in agar⁴². When these E2A-PBX1-expressing cells were injected into nude mice, tumors formed in the area of injection within six weeks⁴². Retroviral transduction of E2A-PBX1 into primary murine bone marrow results in the immortalization of myeloid progenitors and their ability to propagate in GM-CSF-containing media for months. When E2A-PBX1-

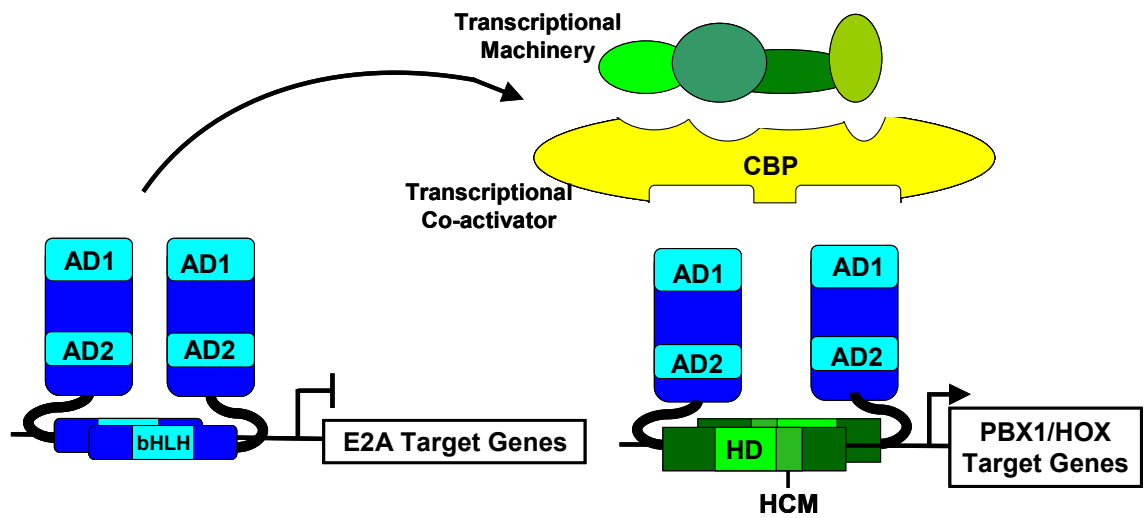
Figure 1-3 Domain structures of E2A-PBX1 proteins and models of transcriptional deregulation

- (A)** Domain structure of the E2A-PBX1 chimeric fusion proteins, E2A-PBX1a and E2A-PBX1b. Both versions of E2A-PBX1 contain the N-terminal portion of the E2A proteins (residues 1-483), which includes both activation domains, AD1 and AD2. E2A-PBX1a and E2A-PBX1b differ at the C-terminus, due to the variation between PBX1a and PBX1b. Both contain the DNA binding HD and the HCM which mediate DNA interactions and HOX heterodimerization, respectively.
- (B)** A model of deregulation by E2A-PBX1, where E2A-PBX1 hijacks the transcriptional co-activator CBP and transcriptional machinery from wild type E2A proteins. This results in the inhibition of wild type E2A target gene expression, and augmentation of PBX1/HOX target gene expression.
- (C)** Another model of deregulation by E2A-PBX1, where E2A-PBX1 competes for wild type PBX1 in heterodimerization with HOX proteins via the intact HD and adjacent HCM. The activation domains of E2A-PBX1 result in the inappropriate expression of PBX1/HOX target genes.

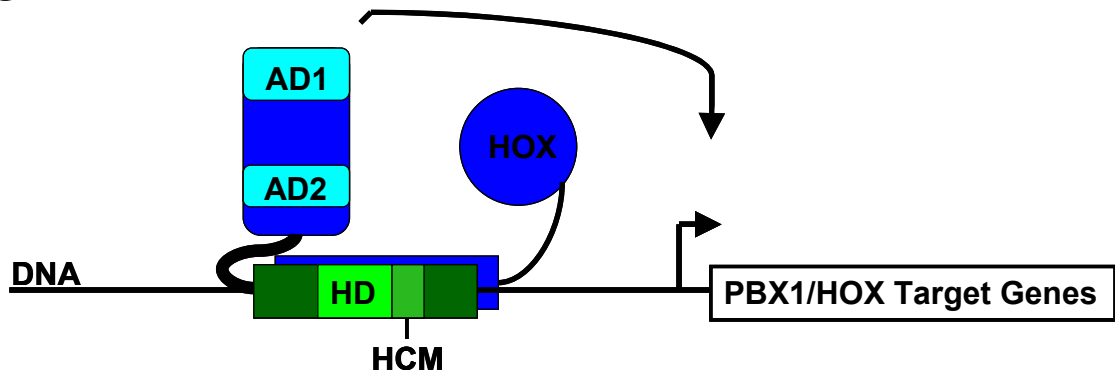
A E2A-PBX1 proteins (E2A-PBX1a and E2A-PBX1b)



B



C



transduced bone marrow cells are transplanted into recipient mice, the animals develop a lethal myeloproliferative disease that resembles acute myeloid leukemia (AML)⁴³. It is unknown why mice injected with E2A-PBX1 develop myeloid and not lymphoid leukemia, as seen in cases of human t(1;19) ALL. It is speculated that perhaps the first cell type to be infected is a myeloid cell, and that a myeloid tumor develops before a lymphoid cell is efficiently infected⁴³. The ability of E2A-PBX1 to block hematopoietic differentiation has been demonstrated by the expression of an estrogen-dependent E2A-PBX1 construct in bone marrow⁴⁴. Propagation of these infected and undifferentiated cells is dependent on a high concentration of estradiol. Removal of estradiol and subsequent inactivation of E2A-PBX1 results in cell cycle arrest and allows the differentiation of these cells into neutrophils and monocytes, indicating alleviation of the block in differentiation caused by E2A-PBX1⁴⁴.

1.5.2 Mechanisms of E2A-PBX1 oncogenicity

To this day, the precise mechanisms of E2A-PBX1 oncogenicity remain uncertain. E2A and PBX1 are both transcription factors with roles in lymphopoiesis, and each imparts important domains to the chimeric protein E2A-PBX1. E2A-PBX1 is considered to act, at least in part, through dominant negative effects on wild type E2A proteins, perhaps by sequestering important co-activator complexes (**Figure 1-3 B**)⁴⁵. This dominant negative effect would decrease wild type E2A function, thus aggravating the deleterious effect of disruption of one of the *E2A* alleles by the translocation. Another plausible mechanism is the aberrant expression of PBX1/HOX target genes downstream of E2A-PBX1. E2A-PBX1 retains DNA binding ability through the PBX1 HD, and the activation domains of E2A offer potent transcriptional activation capabilities. E2A-PBX1 could conceivably compete with wild type PBX1 for a single DNA binding sequence (**Figure 1-3 C**)⁴³. Taken together, these data indicate important and potentially independent contributions by both E2A and PBX1 in the oncogenic potential of E2A-PBX1.

1.6 CBP/p300 proteins

CREB (cyclic AMP response element binding) binding protein (CBP) and p300 are ubiquitously expressed nuclear transcriptional co-regulators. CBP was first discovered as a protein that interacts with CREB consequent to its phosphorylation by protein kinase A⁴⁶, and p300 was initially discovered due to its interaction with the adenovirus E1A oncoprotein⁴⁷. CBP and p300 are encoded on two separate genes, located at the chromosomal loci 16p13.3 and 22q13.2, respectively. They share 63% overall homology at the protein level and show considerable functional similarity. Therefore, although there are functional differences between CBP and p300, they will here be referred to collectively as CBP⁴⁸.

Several domains are highly conserved amongst CBP orthologs in various multicellular organisms, including the CH (cysteine/histidine rich) 1-3, KIX, bromo- (BD), and histone acetyltransferase (HAT) domains (**Figure 1-4 A**)⁴⁹. CBP is recruited to large transcription complexes, where it regulates the transcription of target genes by bridging interactions between DNA binding transcription factors and the transcriptional machinery. Conserved CH domains within CBP interact with RNA helicase A, which recruits RNA polymerase II to commence transcriptional initiation (**Figure 1-4 B**)⁵⁰. The HAT domain is required for efficient transcriptional activity. Histone proteins wrap DNA tightly into nucleosomes, preventing protein binding and impeding the initiation of transcription. HATs catalyze the acetylation of lysine residues on the tails of histone proteins to relax the DNA, allowing for the interaction of proteins to initiate transcription.

1.6.1 Role of CBP in cellular proliferation, development and hematopoiesis

CBP/p300 are implicated in cell proliferation and development; mice with a homozygous deletion at the p300 locus (p300^{-/-}) die within 12 days of embryonic development, presenting defects in neural growth, cellular proliferation and cardiac development⁵¹. CBP interacts with

many transcription factors that have significant roles in hematopoietic differentiation, indicating a possible role in hematopoiesis. Transgenic and knock out studies in murine models have supported this hypothesis; mouse embryos engineered to be homozygous for a truncated form of CBP have many hematopoietic defects⁵². Heterozygosity at the CBP locus (CBP^{+/-}) in mice leads to various defects in hematopoiesis with underlying bone marrow failure⁵³. These *in vivo* findings in murine models indicate that CBP is involved in the regulation of hematopoiesis.

1.6.2 Role of CBP in cancer

CBP interacts with both oncoproteins (Myb, Jun) and tumor suppressor proteins (p53, Rb), is targeted by somatic mutations, and is involved in various chromosomal translocations; cumulative evidence supporting a role for CBP in cancer⁵⁴. Mice engineered to be heterozygous at the CBP locus (CBP^{+/-}) often exhibit loss of heterozygosity at the wild type *CBP* locus which is hallmark of a tumor suppressor gene⁵². This is also supported by the role of CBP in the stability and degradation of p53, through a direct interaction with the tumor suppressor⁵⁴. It is targeted in chromosomal translocations which form chimeric proteins involved in leukemia and lymphoma, such as MOZ-CBP and MLL-CBP⁵⁵. These translocations often result in the deletion of important domains within CBP which can lead to the formation of a hematopoietic malignancy.

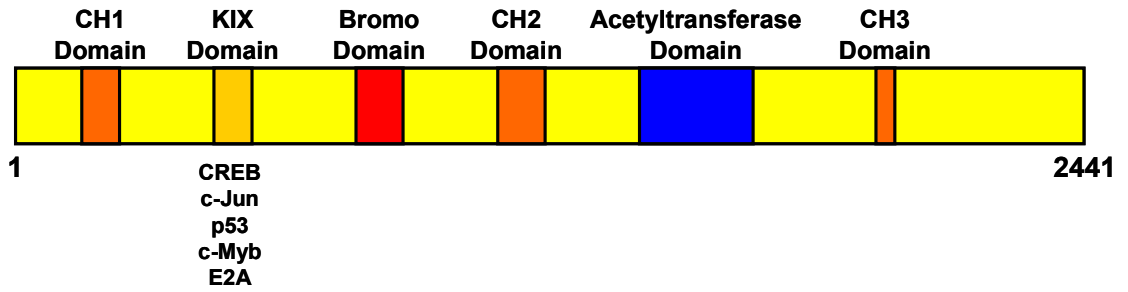
1.6.3 Involvement of the KIX domain in protein-protein interactions

The KIX domain participates in numerous protein-protein interactions and is located centrally towards the N-terminus of CBP (aa 586-666 of murine CBP). The KIX domain is formed by three tightly packed alpha helices, $\alpha 1$, $\alpha 2$, $\alpha 3$, which form a hydrophobic core⁵⁶. The KIX domain forms interactions with many transcription factors, recognizing a conserved LXXLL motif within many of these proteins. Nuclear magnetic resonance (NMR) solution structures of KIX bound to various binding partners have been solved, including KIX:CREB⁵⁶,

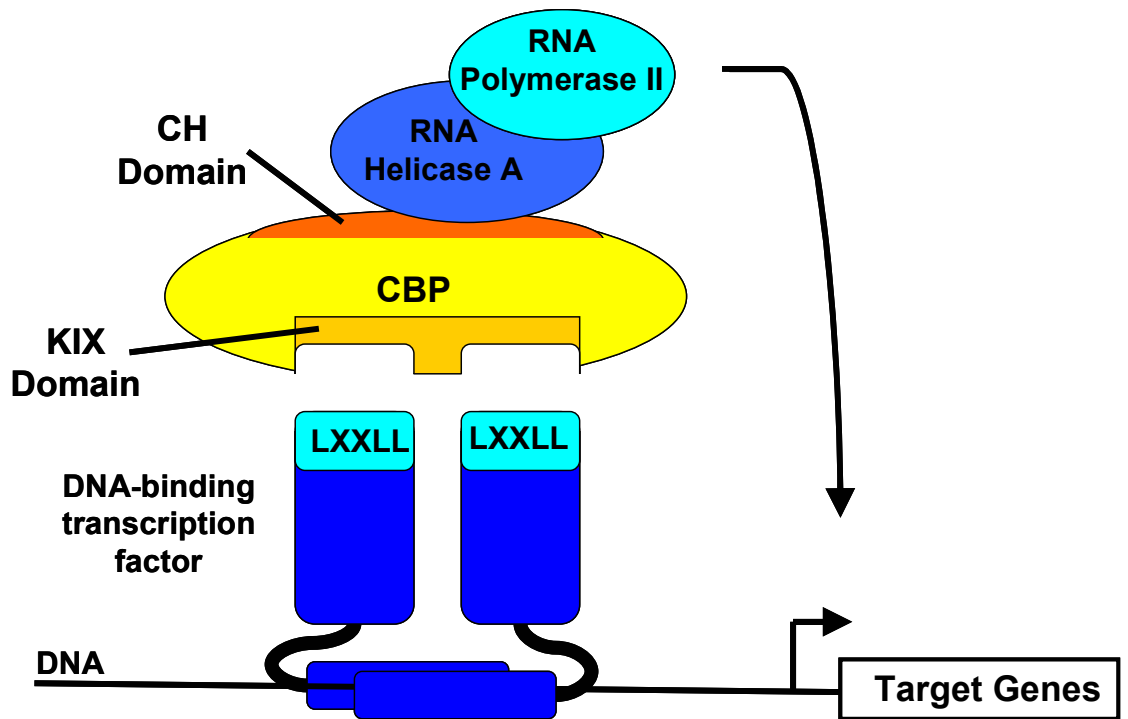
Figure 1-4 Domain structure and functional model of CBP

- (A)** CBP is a large, multimodal protein which contains many highly conserved domains. The CH1, CH2 and CH3 domains are rich in cysteine and histidine residues, and interact with various proteins. The KIX domain forms interactions with many LXXLL-containing proteins including CREB, c-Jun, p53, c-Myb and E2A proteins. The centrally located bromodomain (BD) interacts with various transcription factors in an acetylation-dependent manner. The acetyltransferase (AT) domain acetylates lysine residues in histones which releases DNA to commence transcription.
- (B)** CBP is a transcriptional co-activator which bridges interactions between various proteins to help form a transcriptional co-activator complex. CBP recruits RNA helicase A through the CH domains, which then recruits other proteins such as RNA polymerase II. This multi-protein complex acts to initiate transcription of target genes as dictated by the DNA binding transcription factors.

A Domain structure of CBP



B



MLL:KIX:c-Myb⁵⁷ and KIX:E2A(unpublished data). CREB and c-Myb interact with KIX at the same shallow hydrophobic groove formed by $\alpha 1$ and $\alpha 3$; the transactivation domain of c-Myb recognizes the same site on KIX as the α_B helix of pKID within CREB. Interestingly, the transcription factor MLL interacts with CBP at a separate site on the KIX domain, allosterically potentiating the KIX:c-Myb interaction⁵⁸. E2A interacts with KIX at a site similar to MLL, which is located in a hydrophobic groove adjacent to the CREB/c-Myb binding site, formed by the $\alpha 3$ helix and the loop joining the $\alpha 1$ and $\alpha 2$ helices. Many interactions between transcriptional regulators are mediated by the LXXLL motif³². It is shared amongst CREB, c-Myb, c-Jun and E2A; bulky hydrophobic side groups of the leucine residues within LXXLL interact with hydrophobic grooves formed by the KIX domain.

1.7 Focal point of E2A-PBX1:CBP interaction

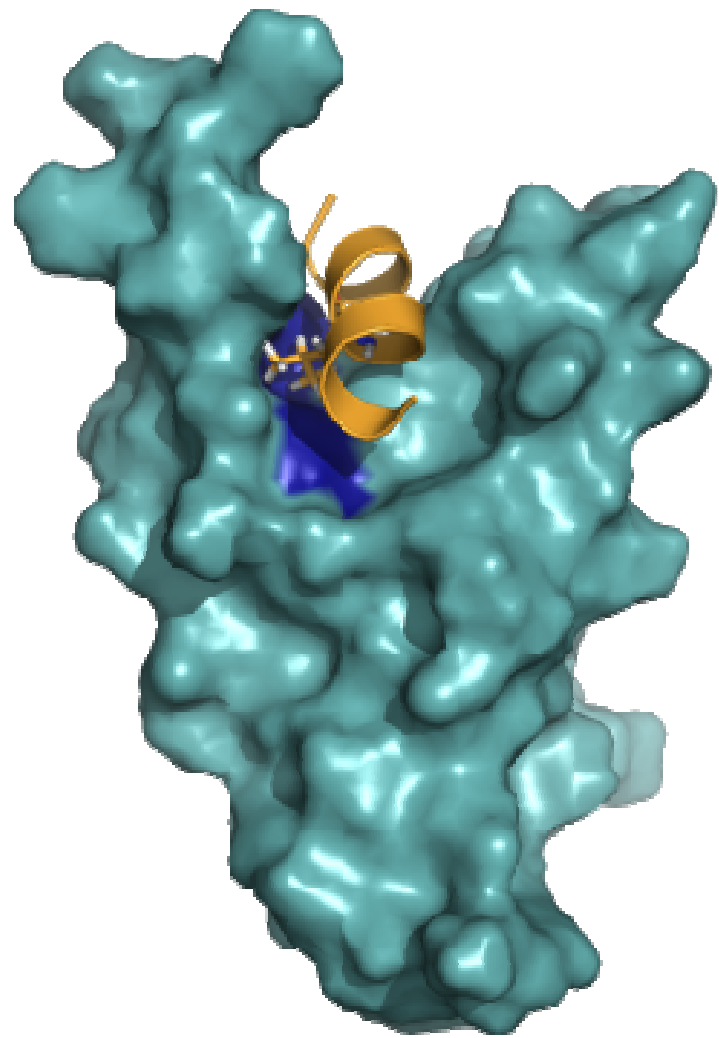
A functional interaction between E2A and CBP has previously been demonstrated by co-transfection experiments with an IgH reporter plasmid in NIH 3T3 cells; CBP acted synergistically to enhance reporter expression by E2A²³. Each AD within E2A has a portion that forms an α -helix (aa 16-23 within AD1 and aa 397-405 within AD2), and both are essential for transcriptional activation. The LXXLL motif that contributes to the hydrophobic KIX interaction is found within the helical portion of AD1, spanning residues L¹⁶-L²⁰. In a pull down assay, Bayly *et al* were able to show that both AD1 and AD2 were involved in the KIX interaction¹. Deletion of both ADs completely eliminated the interaction, and both α -helical domains within AD1 and AD2 directly interact with KIX. Retroviral infection of E2A-PBX1 into primary bone marrow cells results in increased proliferation and cellular immortalization in cultured media³¹. Retroviral infection with E2A-PBX1 lacking AD1 reduced cellular proliferation and immortalization, an effect that was diminished further by the deletion of both

AD1 and AD2¹. More specifically within AD1, engineering a point mutation at position 20 by exchanging the leucine for an alanine (L²⁰A) resulted in a substantial inhibition of the CBP interaction in a pull down assay³¹. Retroviral transduction of the L²⁰A mutant into primary bone marrow cells did not result in cellular immortalization, unlike wild type E2A-PBX1³¹. Similarly, the injection of E2A-PBX1-transduced bone marrow cells into lethally irradiated mice leads to the development of a myeloproliferative disease and death within 40 days post-transplantation. However, when mice are injected with the L²⁰A mutant of E2A-PBX1 they survive past 100 days. Taken together, these data demonstrate the crucial importance of a single residue within E2A-PBX1, L²⁰, in both the interaction between E2A-PBX1 and CBP and the development of cancer in mice. Using NMR spectroscopy, our lab has shown that the hydrophobic side chain of this L²⁰ residue fits into a hydrophobic groove formed by the KIX domain of CBP (unpublished data, Chris Denis)(**Figure 1-5**). Since this interaction relies on a focal point of contact, it might be susceptible to small molecule antagonism.

High throughput screening for small molecule inhibitors of protein-protein interactions is relatively new in cancer research. The use of automated robots, able to subject an assay to up to millions of compounds in a short time period, has made high throughput screening a quick and straightforward first step in the drug discovery process. Binding assays based on the fluorescence anisotropy (FA) of a fluorescently tagged peptide are highly sensitive, reproducible, and have become one of the most popular methods used for high throughput screening⁵⁹. FA can accurately detect changes in the molecular weight of a fluorescently labeled peptide, which is indicative of an interaction. Several groups have been successful in finding inhibitors of protein-protein interactions using high throughput screening of small molecule libraries, if the interactions rely on a small focal point and not a large surface area⁶⁰. Roehrl *et al* were successful in finding a small molecule inhibitor for a protein-protein interaction with an FA-

Figure 1-5 The L20 residue of E2A-PBX1 fits into a hydrophobic cleft in the KIX domain

Using NMR spectroscopy, our lab has solved the structure of the KIX domain (light blue) bound to the helical portion of AD1 within E2A (orange). The L²⁰ hydrophobic side chain is displayed, which interacts with hydrophobic residues within the KIX domain (dark blue). E2A interacts with a cleft formed by the α 3 helix and the loop joining the α 1 and α 2 helices within KIX.



based binding assay⁶¹. Other groups have used different types of binding assays to successfully find small molecule inhibitors of protein-protein interactions, such as assays based on flow cytometry⁶².

1.8 Hypothesis

The oncogenic interaction between E2A-PBX1 and CBP is susceptible to inhibition by small molecules. Such inhibitors will antagonize the leukemic effects of E2A-PBX1, serve as probes for the elucidation of E2A-PBX1 function, and act as lead compounds in developing improved treatments for ALL.

1.9 Experimental objectives

- (1) Design and validate a cell-free binding assay based on FA to monitor the interaction between E2A-PBX1 and CBP;
- (2) Perform high-throughput screening using this assay in order to identify compounds in small molecule libraries that disrupt the interaction;
- (3) Validate positive hits identified by the high throughput screening and characterise the validated hits using biophysical and biochemical techniques;
- (4) Begin to investigate the effects of validated small molecules in cell-based assays using cultured cells that dependent on E2A-PBX1 oncogenicity for survival.

Chapter 2

Materials and Methods

2.1 Cell-free binding assay

2.1.1 FITC-E2A

An N-terminal fluorescein (FITC) labeled- fragment of E2A comprising residues 11-24 and containing the highly conserved LXXLL motif (N-GTDKELSDLLDFSM-C, LXXLL underlined) was ordered from the Peptide Synthesis/Purification Facility at McGill University. The peak excitation and emission wavelengths of the FITC-E2A molecule were experimentally determined to be 492 nm and 523 nm, respectively. These wavelengths were used in all subsequent experiments.

2.1.2 Expression and purification of recombinant KIX

pET21a(+) plasmids expressing the KIX domain of CBP/p300, residues 586-671 (gift from Dr. Peter Wright, Scripps Research Institute, La Jolla, California), were transformed into *Escherichia coli* by electroporation. A bacterial culture was grown in Terrific Broth and recombinant protein expression was induced by addition of isopropyl β -D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. The culture was grown overnight at 24° C and the pelleted bacteria were re-suspended in Binding buffer (25 mM Tris, 250 mM NaCl, pH 8.0) containing 8 M urea. The bacterial cells were then lysed by sonication on ice, for five separate twenty second periods at an amplitude of 35%, each separated by a few seconds of gentle swirling. The resulting lysis suspension was ultracentrifuged at 40,000 rpm (70 Ti Rotor, Beckman Coulter) for one hour. The supernatant was equilibrated on a nickel affinity column for a minimum of thirty minutes, and the KIX was isolated through the interaction between endogenous histidine residues and the nickel affinity column. After allowing the supernatant to

evacuate the column, it was washed with Binding buffer, and eluted with Binding buffer containing 300 mM imidazole. The elution fraction was further purified to homogeneity via size exclusion chromatography and anionic exchange chromatography. Purified KIX was dialyzed against buffer containing 20 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 6.0, and 1 mM beta-mercaptethanol (β -Me) and stored at -80°C . Samples of KIX were analyzed on an 8% polyacrylamide gel to verify the presence of protein.

2.1.3 Fluorescence anisotropy binding assay

All fluorescent samples were analyzed using the FluoroLog Tau-3 Fluorescence Lifetime Spectrofluorometer with anisotropy capability (HORIBA Jobin Yvon Inc., Edison, NJ) in the Queen's University Protein Function Discovery Facility. All samples were dissolved in buffer containing 20 mM MES, pH 6.0, 1 mM β -Me. Samples were contained in a quartz cuvette with a stir bar, which allowed constant mixing during the titration. A 1 μM FITC-E2A sample was titrated against increasing concentrations of KIX, from 0 μM to 70 μM . KIX samples were prepared and appropriate volumes to be titrated into FITC-E2A were calculated prior to the experiment. FA readings were recorded in triplicate after each injection of KIX, and the average value of each point was graphed against KIX concentration. Data were fit to a hyperbolic equation as previously derived by Roehrl *et al* and the K_D value was determined from this equation⁵⁹. Titrations were performed twice to ensure reproducibility of results.

2.1.4 Fluorescence anisotropy calculation

The FA is calculated based on a ratio of the parallel (||) and perpendicular (\perp) emitted fluorescence (I) of the sample, subsequent to excitation with plane polarized light. The denominator represents the sum of the parallel fluorescence and two times the perpendicular

fluorescence, thus the equation takes into account all three dimensions (x, y, z), and is normalized to the total fluorescence of the sample. FA is therefore concentration independent.

$$\mathbf{FA} = \frac{\mathbf{I}_{\parallel} - \mathbf{I}_{\perp}}{\mathbf{I}_{\parallel} + 2\mathbf{I}_{\perp}} \quad \text{screening}$$

2.2 High throughput

Samples of 2 μM FITC-E2A and 30 μM KIX in buffer containing 20 mM MES, pH 6.0, and 1 mM β -Me were brought to the Samuel Lunenfeld Research Institute in Toronto. The multidrop 384 (Thermo Electron Corp.), a robot for dispensing bulk reagents, was used to dispense 12.5 μL of both 2 μM FITC-E2A and 30 μM KIX samples into each well of a 384-well plate to achieve final concentrations of 1 μM and 15 μM , respectively. Initial FA of each well was detected using the CRS Dimension4TM modular automation system (Thermo Electron Corporation). Each small molecule was dispensed to a final concentration of 8 μM in their respective wells. After 15 minutes the FA was measured. Small molecules were given a B score (section 2.2.1), and those resulting in a decrease in FA greater than three standard deviations below the mean were considered positive hits. Readings were performed twice to ensure reproducibility of the changes in FA. Small molecules resulting in a reproducible decrease in FA were used for further analysis.

2.2.1 B score calculation

Small molecules in the libraries were statistically analyzed with B scores, calculated using a computer algorithm based on the equation:

$$\mathbf{B\ Score} = \frac{\mathbf{y}_{ijp} - (\hat{\mu} + \hat{\mathbf{R}}_{ip} + \hat{\mathbf{C}}_{jp})}{\mathbf{MAD}_p}$$

where the y_{ijp} represents the observed result for the i^{th} row, j^{th} column and p^{th} plate, μ^{\wedge}

represents the plate average, R_{ip}^{\wedge} represents the measurement offset for row i on plate p , C_{jp}^{\wedge} represents the measurement offset for column j on plate p and MAD_p represents the median absolute deviation of plate p ⁶³. In this equation, the numerator accounts for positional effects and inter-plate changes, while the denominator accounts for residual plate variability, after row and column effects are accounted for⁶⁴.

2.3 Validation of positive hits

The ability of each positive hit to reproducibly decrease FA was measured in the Queen's Protein Function Discovery facility. The FA associated with 1 μ M FITC-E2A and 15 μ M KIX in 20 mM MES buffer, pH 6.0, with 1 mM β -Me was measured before and after the addition of 8 and 11 μ M of each positive hit. Small molecules causing a reproducible decrease in FA were considered validated hits. The endogenous basic fluorescence of 1 μ M samples of each validated hit was then detected at an excitation wavelength of 492 nm and an emission wavelength of 523 nm. Small molecules with intrinsic fluorescence equal to or greater than FITC-E2A were titrated against KIX to determine a possible interaction. Intrinsically fluorescent small molecules that did not demonstrate an interaction were discarded from further analysis. Non-fluorescent compounds were titrated into FITC-E2A to determine a possible interaction with the E2A peptide.

2.4 Preparation of nuclear extracts

Nuclear extracts were prepared from RCH ACV cells, a pre-B ALL cell line expressing E2A-PBX1, using the Dignam and Roaeder method⁶⁵. Briefly, approximately 4×10^7 RCH ACV cells were grown to confluency in 10 cm tissue culture dishes in RPMI media with 10% fetal bovine serum (FBS). Cells were harvested at 1×10^6 cells/mL, pelleted, and washed twice in PBS. The washed cell pellet was re-suspended in 5 packed cell volumes of Buffer A (10 mM HEPES-KOH pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, protease inhibitors) and homogenized

using approximately 50 strokes in a dounce homogenizer. Efficient lysis of cellular membranes was confirmed using trypan blue for the presence of stained nuclei. Nuclei were pelleted, re-suspended in Buffer B (20 mM HEPES-KOH pH 7.9, 25% v/v glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA pH 8.0, 0.5 mM DTT, protease inhibitors) and stirred at -4°C for one hour. The solution was pelleted and supernatant was dialyzed for a minimum of five hours at 4°C against buffer D (20 mM HEPES-KOH pH 7.9, 20% v/v glycerol, 0.1 M KCl, 0.2 mM EDTA pH 8.0, 1 mM DTT, protease inhibitors). The dialyzed extract containing nuclear proteins was centrifuged to remove precipitants and aliquoted to be stored at -80°C.

2.5 Pull-downs using nuclear extracts

Ten µg of GST-E2A (aa 1-483) fusion protein was incubated with 50 µL pre-swollen glutathione sepharose 4B beads (GE Health Care) in PBS containing 1% Triton X-100 to a final volume of 200 µL for 2 hours at 4°C on a nutator. The beads were pelleted by centrifugation at 5000 rpm for 5 minutes, and washed in HEGN buffer (20 mM HEPES-KOH pH 7.9, 0.1 mM EDTA pH 8.0, 10% v/v glycerol, 0.1% NP-40, 1 mM DTT, protease inhibitors). Non-specific protein binding was blocked by incubating the beads in 1 µg/µL bovine serum albumin (BSA) and 0.1 M KCl for a minimum of one hour. The 50 µL beads were pelleted and subsequently incubated with 1 mg RCH ACV nuclear extract to a final concentration of 1 mL in HEGN buffer. Incubation with nuclear extract was overnight at 4°C on a nutator. The next day, glutathione beads and bound proteins were washed three times in HEGN buffer containing 200 mM NaCl. Pelleted beads were then re-suspended in 50 µL electrophoresis sample buffer containing β-Me and proteins were eluted by boiling for a minimum of five minutes. Samples were run on a 6% SDS-PAGE gel and the presence of bound protein of nuclear extract origin was determined by Western blotting.

2.6 Western blotting and antibodies

Proteins from the SDS-PAGE gels were transferred to a nitrocellulose membrane (Perkin Elmer Life and Analytical Sciences) in a transfer apparatus (Bio-Rad) at a constant 70V for 90 minutes on ice. The membranes were subsequently blocked for a minimum of one hour in PBS containing 0.1% Tween (PBS-Tween) and 5% non-fat dried milk. The membrane was then incubated with primary antibody in PBS-Tween containing 3% non-fat dried milk and 0.02% sodium azide overnight at 4°C while being slowly rocked. Membranes were then washed three times in PBS-Tween for approximately 15 minutes per wash, and incubated with secondary antibody conjugated to horseradish peroxidase for a minimum of one hour at room temperature, while being rocked. Membranes were washed three times in PBS-Tween for approximately 20 minutes per wash, and the membrane was then subjected to chemoluminescence by incubating in equal volumes of oxidizing reagent and enhanced luminal reagent (Perkin Elmer Western Lightning Chemoluminescence Reagent Plus) for one minute. The membranes were then used to expose photographic film. Primary antibodies were diluted to a final concentration of 1:4000 of the original sample volume, and included p300 rabbit polyclonal IgG; E2A (Yae) mouse monoclonal IgG; and c-Abl rabbit polyclonal IgG (all from Santa Cruz Biotechnology). Secondary antibodies were diluted to a final concentration of 1:10000 of the original sample volume, and included goat α -rabbit horseradish peroxidase conjugated and goat α -mouse horseradish peroxidase conjugated (from Jackson Laboratories).

2.7 NMR spectroscopy

NMR experiments were performed on a sample of 500 μ M of recombinantly expressed $^{13}\text{C}/^{15}\text{N}$ KIX in 20mM MES, pH 6.0, 1 mM β -Me. HSQC datasets were recorded on a Varian INOVA 500 MHz spectrometer equipped with a cryoprobe. Each compound was added to uniformly $^{13}\text{C}/^{15}\text{N}$ -labeled KIX to a concentration of 5 mM.

2.8 Retroviral transductions of bone marrow cells

Bone marrow was harvested from femurs of BALB/c mice on day one of the experiment and re-suspended in prestimulation mix (IMDM supplemented with 15% fetal bovine serum (FBS), 10 ng/mL IL-3, 10 ng/mL IL-6 and 75 ng/mL murine stem cell factor; all cytokines were purchased from BioShop Canada Inc.). SV293T cells were plated at a density of 3×10^6 cells in 100 mm dishes in DMEM supplemented with 10% FBS the day before transfection. Media was changed one hour before transfections and DNA was precipitated using the calcium phosphate precipitation protocol⁶⁶. Each plate of SV293T cells was co-transfected with either MIEV-EP1B, MIEV-L20A or MIEV empty vector, in addition to MCV-ecopac (ecotropic packaging) plasmids (the MIEV retrovirus was a generous gift from Dr. Peter Greer; wild type and L20A versions of E2A-PBX1 were cloned into the vector by Richard Bayly. MCV-ecopac was a generous gift from Dr. David Sykes). On day 2, the media was changed on the SV293T cells and the bone marrow cells were re-suspended in fresh pre-stimulation mix. The next day, the SV293T cells were exposed to 15 Gy of ionizing radiation and the bone marrow cells were split between the Petri dishes containing packaging cells in pre-stimulation mix with 8 μ g/ml polybrene. On day 4, the bone marrow cells were re-suspended in fresh pre-stimulation mix with polybrene and co-cultured with the packaging cells for another day. The next day, the bone marrow cells were removed and re-suspended in IMDM supplemented with 10% FBS and 10 ng/mL granulocyte macrophage-colony stimulating factor (GM-CSF) in fresh 25 cm flasks and the bone marrow-derived cells were propagated under these conditions thereafter.

2.9 MTT cytotoxicity assays

On day 1, cells were plated at a density of 8×10^4 cells in 100 μ L of media per well in 96 well plates. The next day, the drugs were diluted and 100 μ L of desired drug concentration was added to the wells in quadruplicate. The cells were incubated at 37 °C in a CO₂ incubator for 72

hours. On day 5, 25 μ L of MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] solution (2 mg/mL in PBS) was added to each well and cells were then incubated for 3 hours at 37 °C. After three hours, 100 μ L of 1N HCl/iPrOH(1:24) was added to each well to stop the reaction, and mixed vigorously. The plates were read using the BioTEK ELx 800 microplate reader at 570 nm and the average of the concentration points were plotted against drug concentration. EC₅₀ values were determined by normalizing the data and plotting % control absorbance vs drug concentration.

2.10 Mammalian two-hybrid assays

The day before the experiment, SV293T cells were plated at a density of 1.7×10^5 cells per well into 12-well plates, in DMEM media supplemented with 10% FBS. The next day, the SV293T cells were transfected with mammalian expression and reporter plasmids by the calcium phosphate precipitation method⁶⁶. Into each well, 0.01 μ g of VP16 or GAL4 wild type or fusion plasmid was added, conferring the expression of either VP16, VP16-E2A, GAL4, GAL4-KIX, or a control GAL4-VP16 fusion protein. Also added into each well was 0.1 μ g of renilla plasmid and 0.7 μ g of 5X GAL4 luciferase reporter plasmid. Twenty-four hours after transfection, fresh DMEM media supplemented with 10% FBS was substituted and varying concentrations of small molecules were added to the appropriate wells. Cells were harvested 24 hours after the addition of fresh media/small molecules, and washed in PBS. Cells were lysed in passive lysis buffer (Promega) for a minimum of 30 minutes at room temperature on a nutator. Twenty μ L of each lysate was analyzed using dual-luciferase assay reagents (Promega), on an LB96V MicroLumat Plus luminometer (EG & G Berthold Ltd., Bundoora, Australia). Each transfection and condition was performed in triplicate, and the results were averaged. The luciferase values of each sample were normalized with their renilla values, to standardize each value. Values were plotted as a

percentage of maximum luciferase expression, which was chosen to be E2A:KIX alone or the control GAL4-VP16 fusion protein.

Chapter 3

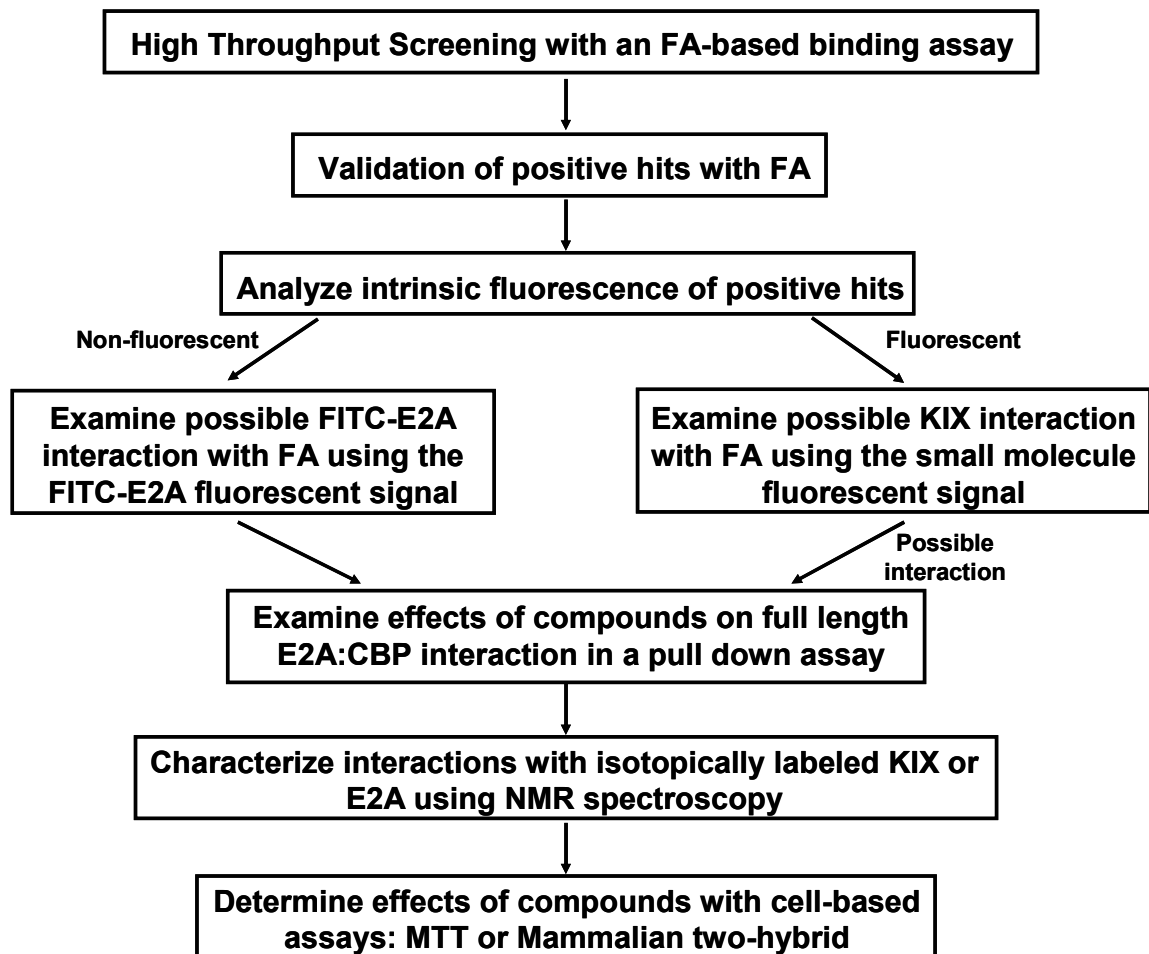
Results

3.1 Formulation of a step-wise protocol for finding small molecule inhibitors

An efficient and rational step-wise protocol was developed for finding and evaluating small molecule inhibitors of the E2A-PBX1:CBP interaction (**Figure 3-1**). The FA-based assay was developed to screen small molecule libraries with high throughput screening to find potential small molecule inhibitors to analyze. Each step thereafter involved a different analysis of the small molecules, and provided information to make a rational decision on whether to pursue further analysis of the compounds. Positive hits from the screens were considered validated if they were able to cause a reproducible decrease in FA. Intrinsically fluorescent small molecules, which added fluorescence to the sample and interfered with the FA values, were eliminated unless they demonstrated a possible interaction with KIX. Non-fluorescent small molecules were also analyzed using FA for possible interactions with FITC-E2A. Pull down assays eliminated compounds which were unable to inhibit the full length E2A:CBP interaction, and NMR spectroscopy provided information on small molecule interactions with the KIX domain, and could also be used for E2A interactions. Cell-based assays then provided information on the effects of the compounds on living cells. Cytotoxic effects were examined using the MTT assay and effects of the compounds on the KIX:E2A interaction in live cells were examined with the mammalian two-hybrid assay. This chronological series of assays could be used in the future to find small molecule inhibitors of the E2A-PBX1:CBP interaction, or any other protein-protein interaction found to be critically dependent on a small area of contact.

Figure 3-1 Step-wise protocol for the discovery of small molecule inhibitors

Beginning with the development of an FA-based cell-free binding assay, this flow chart outlines the chronological series of experiments involved in the analysis and characterization of small molecules beginning with high throughput screening. Each step represents an assay or type of evaluation of the small molecules, and compounds that fail at any particular stage are removed from further analysis.

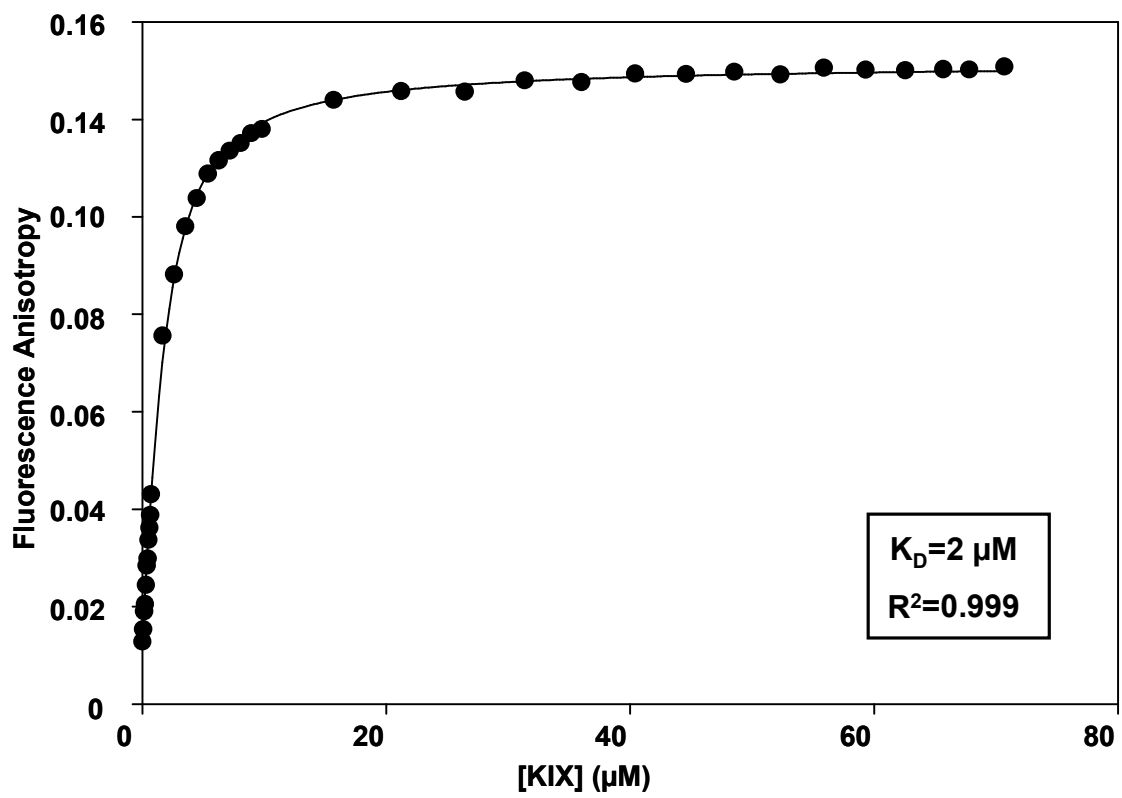


3.2 Development of a cell-free binding assay based on fluorescence anisotropy

In order to screen for small molecule inhibitors, an assay was required that would detect the interaction between E2A and KIX in a manner that was simple and amenable to automation for high-throughput screening. A small portion of E2A (aa 11-24) was fluorescently tagged at the N-terminus with a fluorescein (FITC) label. Different buffer conditions were tested in order to determine optimal parameters for the assay, including different pH values, salt concentrations and buffer concentrations. Binding curves for the FITC-E2A:KIX interaction were obtained for each of the different buffer conditions, and the buffer allowing the tightest interaction was chosen. This buffer contained 20 mM MES pH 6.0. FA is a measure of rate of tumbling of molecules in solution that is based on the polarization of light emitted from a fluorescent sample subsequent to excitation by polarized light (**section 2.1.4**). The degree of rotation of a fluorescent molecule in the time between excitation of the sample and detection of emitted light can be measured using FA, and is indicative of the size of the molecule. Small molecules that rotate quickly in solution have a low level of FA whereas larger molecules that rotate slowly have high levels of FA. FITC-E2A in MES buffer alone had an FA value of approximately 0.03, an expected value for a small fluorescent peptide. When increasing concentrations of recombinantly purified KIX were titrated into the FITC-E2A, the FA increased abruptly as the KIX bound to the E2A (**Figure 3-2**). The slope of the interaction was relatively steep and FA values eventually reached a plateau after approximately 20 μ M KIX was added, indicating that at this point the KIX had completely saturated the FITC-E2A peptide. Data from this titration were fit to a hyperbolic equation previously derived by Roehrl *et al*⁵⁹ and the dissociation constant (K_D) of the interaction was determined to be 2 μ M.

Figure 3-2 A fluorescently-labeled E2A peptide binds to KIX with a 2 μ M K_D

Titration of recombinantly expressed KIX into FITC-E2A results in an increase of FA from 0.03 to approximately 0.15. The steep curve indicates that the interaction is strong, and the KIX molecules are binding to the FITC-E2A quickly to cause an abrupt increase in FA. The plateau indicates that the KIX peptides have saturated the FITC-E2A. Data were fit to a hyperbolic equation, demonstrating that E2A and KIX interact with a K_D of 2 μ M.



3.3 Validation of the cell-free binding assay

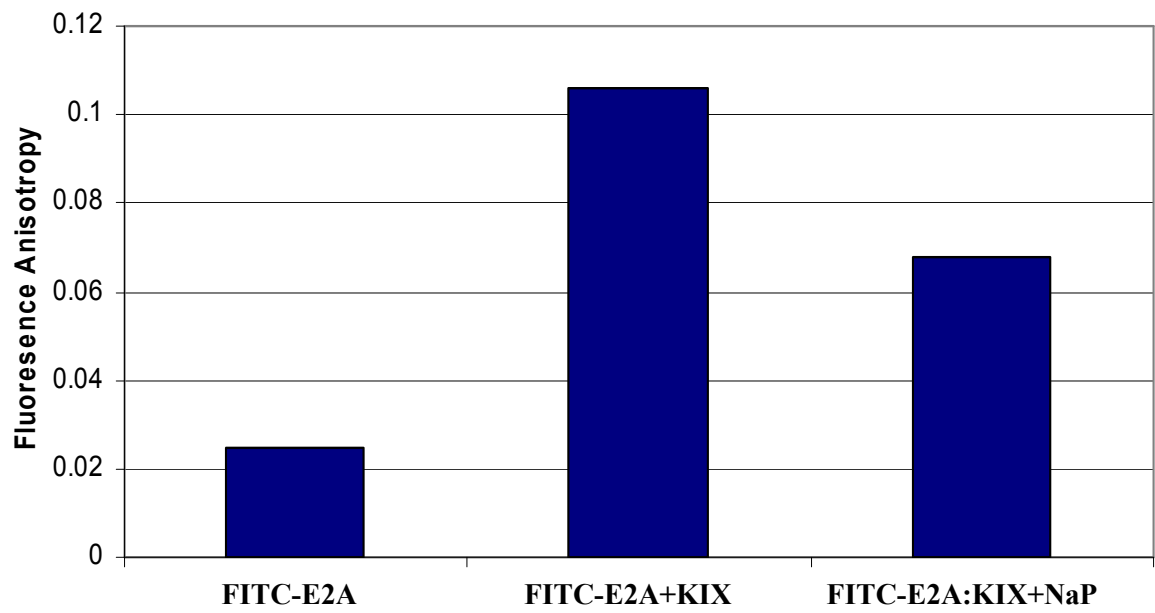
In order for this cell-free assay to be used efficiently to screen for small molecule inhibitors of the interaction, it had to be validated to ensure that it could properly detect disruption of the interaction. The compound naphthol phosphate (NaP) was previously determined to bind to the KIX domain at a site adjacent to the E2A binding site, inhibiting interactions with other KIX binding partners such as CREB⁶⁷. It was predicted that there could potentially be some overlap between the CREB binding site and the E2A binding site on the KIX domain, and that NaP could therefore indirectly inhibit the E2A:KIX interaction to validate the assay. Since high levels of FA indicate bound E2A:KIX, and low levels indicate unbound E2A, we predicted that the addition of NaP would result in a decrease in FA. Indeed, when NaP was added to the E2A:KIX sample, it resulted in a reduction in FA (**Figure 3-3**). Therefore, these results validated this FA assay as a means of detecting disruption of the E2A:KIX interaction by small molecule compounds.

3.4 High throughput screening of small molecule libraries with the cell-free assay

The next step was to perform high throughput screening of small molecule libraries using the binding assay. Three small molecule libraries were screened by technicians at the Samuel Lunenfeld Research Institute in 384-well plates which contained 1 μM E2A and 15 μM KIX in a volume of 25 μL in each well. The three libraries screened were Prestwick, Lopac and Chembridge, which contain 1120, 1280 and 10,000 compounds, respectively. Compounds from the Prestwick and Lopac libraries are marketed drugs with known biological and pharmacological activities while compounds from the Chembridge library are drug-like molecules that have been designed computationally. The resultant FA values were detected after addition of each compound to the wells containing E2A:KIX to a final concentration of 8 μM . The results on FA were represented by a B score, which is a statistical measure of standard deviation (**section 2.2.1**).

Figure 3-3 Naphthol phosphate indirectly inhibits the E2A:KIX interaction

NaP, initially discovered as an inhibitor of the interaction between the KIX domain and CREB, was used to validate the FA-based binding assay. FITC-E2A alone had a low level of FA, which increased upon the titration of KIX into the sample. Addition of NaP to FITC-E2A:KIX caused a reduction in FA, indicative of unbound FITC-E2A and efficient inhibition of the interaction.



The compounds which resulted in a decrease in FA below three standard deviations from the mean were considered positive hits (**Figure 3-4**). From each of the Prestwick and Lopac libraries, 12 positive hits were obtained (denoted P1 to P12 and L1 to L12, respectively) and from the Chembridge library 19 positive hits were obtained (denoted C1 to C19) (**Table 3.1**). Samples of these compounds were further analysed at Queen's University.

3.5 Validation of positive hits by fluorescence anisotropy

Due to the nature of high throughput screens, in which typically hundreds to hundreds of thousands of compounds are analyzed simultaneously by robotics at an extremely rapid rate, and due to the nature of our FITC-E2A:KIX binding assay, the possibility existed of false positives arising from the screen. In order to separate the false positives from the true positives, the hit compounds were individually re-assessed for their effects on the FA resulting from the E2A:KIX interaction. Each compound, at concentrations of 8 μM (data not shown) and 11 μM (**Figure 3-5**), was mixed with FITC-E2A (1 μM) and KIX (15 μM). Thus, by repeating the validation screen using the same conditions as the high throughput screen, we eliminated chances that the compounds did not cause a reproducible decrease in FA due to different assay conditions.

Of the 24 hits from the first two libraries, six caused a reproducible drop in FA: P1, P2, P9, and L1-L3 (**Figure 3-5**). From the third library screen, C1, C2, C9, and C14, caused a decrease in FA (**Table 3.1**). Compound P2 and compound L1 are the same compound, hereafter referred to as compound L1.

3.5.1 Endogenous fluorescence of small molecules

In our assay, FA is calculated based on the fluorescent signal emanating from the FITC molecule attached to the E2A peptide, detected as both parallel and perpendicular emitted polarized light (**section 2.1.4**). FITC-E2A alone has a low FA, which increases upon binding

Figure 3-4 Results from the Prestwick library high throughput screen

Data from the high throughput screen are plotted and analyzed in a graph of sample identification number vs. B score, a measure of standard deviation. Each dark blue dot represents one of the compounds from the small molecule library. Compounds that resulted in a change in B score indicative of a decrease in FA below three standard deviations from the mean (as indicated by the lower light blue line) were considered positive hits. These compounds were brought back to the Protein Function Discovery Facility at Queen's University to undergo further analyses.

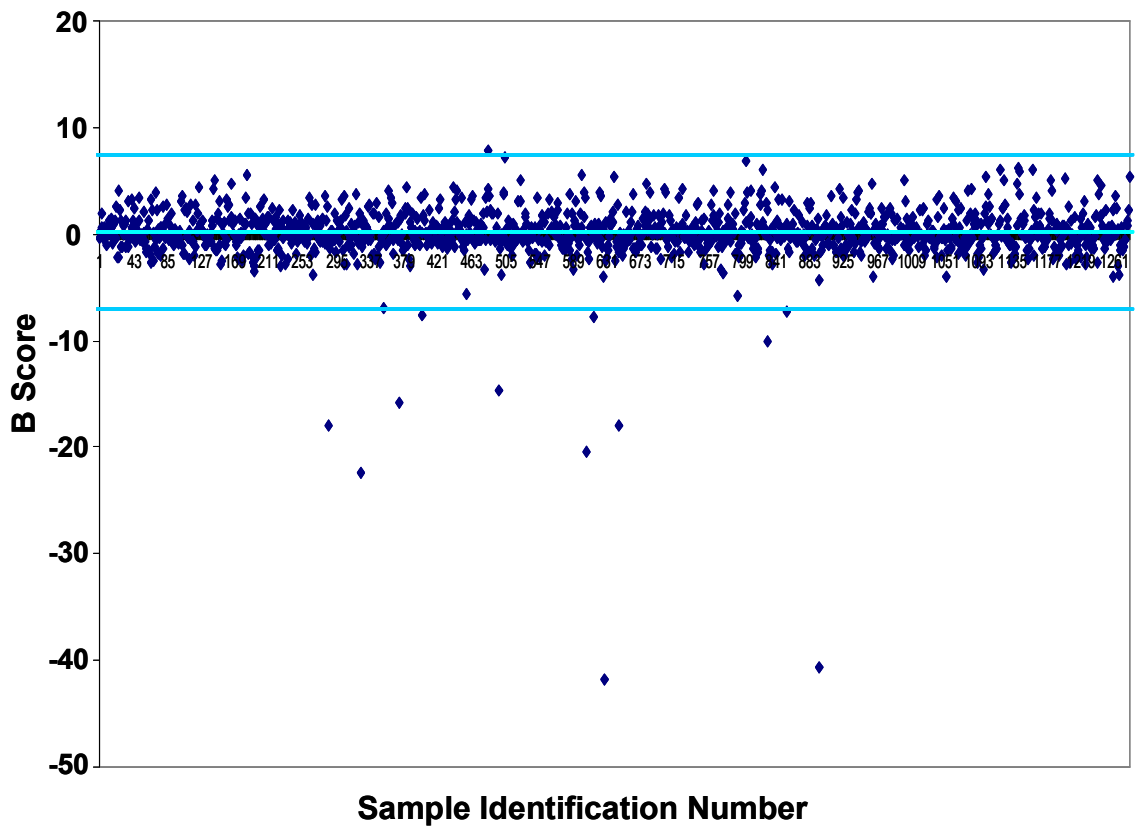


Table 3-1 Summary of positive hits from high throughput screening

(A) Results of the high throughput screen and the process of validation of the positive hits.

Each validation step includes the elimination of compounds which do not meet the requirements for an E2A-PBX1:CBP inhibitor.

(B) Figure legend.

A

Library	# of small molecules	Compound description	Positive hits	FA validated hits	Intrinsically fluorescent validated hits	Pull down validated hits	Cytotoxicity assay validated hits
Prestwick	1120	90% marketed drugs, 10% bioactive alkaloids	12 (P1 to P12)	3 (P1, P2, P9*)	1 (P2) *Non-fluorescent P9 not included	1 (P9)	1 (P9)
Lopac	1280	Marketed drugs, pharmacologically active compounds	12 (L1 to L12)	3 (L1, L2, L3)	1 (L1)	0	0
Chembridge	~10,000	Drug-like compounds, reflect known pharmacophores	19 (C1 to C19)	4 (C1, C2, C9, C15)	1 (C2)	0	0

B

Positive hits	Compounds which resulted in a decrease in FA of FITC-E2A:KIX in the high throughput screening
FA validated hits	Compounds which were able to cause a reproducible decrease in FA
Intrinsically fluorescent validated hits	Intrinsically fluorescent compounds which demonstrate an interaction with KIX by FA using the fluorescent signal of the compounds
Pull down validated hits	Validated hits able to inhibit the full length GST-E2A:CBP interaction
Cytotoxicity assay validated hits	Validated hits with cytotoxic effects on E2A-PBX1-expressing cells

to KIX due to the vast increase in size and consequent decrease in tumbling rate of the FITC-E2A peptide. Following addition of a non-fluorescent compound, reduction in FA is indicative of interruption of the interaction since inhibition of KIX binding increases the fraction of unbound FITC-E2A, which rotates faster alone in solution with a lower FA. However, with the exception of compound P9, all of the small molecule hits had intrinsic fluorescence in the range of wavelengths used for FITC detection. When tested in equimolar concentrations, all of these compounds demonstrated intrinsic fluorescence at levels above that of FITC-E2A (data not shown). However, in the high throughput screening, the small molecules were tested for efficacy at concentrations eight times greater than that of FITC-E2A. The fluorescence of the small molecules therefore significantly adds to the parallel and perpendicular emitted fluorescence values of FITC-E2A, which are used to calculate FA. This makes it difficult to interpret what is causing the reduction in FA values. Small unbound fluorescent compounds have low levels of FA, values that are comparable to the FA of unbound FITC-E2A. Therefore, the reduction in FA following addition of these fluorescent compounds cannot be attributed to their ability to inhibit the interaction. Since the small molecules were added to the FITC-E2A:KIX sample at much higher concentrations than FITC-E2A, the FA calculations were based largely on the signals from the small molecules, which have low FA values irrespective of potentially interacting with FITC-E2A or KIX to inhibit the interaction. Thus, fluorescent hits were ruled out as false positives unless they demonstrated a possible interaction with KIX. This interaction was shown by an increase in FA of the compounds upon addition of KIX, using the intrinsic fluorescence of the compounds to calculate FA.

The addition of KIX to two of the compounds, L1 and C2, caused an increase in FA when calculated based on the fluorescence of the compounds, suggesting that these compounds bind directly to the KIX domain (**Figure 3-6**). Fluorescent hits with no evidence of a KIX

Figure 3-5 Validation of positive hits individually by fluorescence anisotropy

Data shown are for the validation of the positive hits from the high throughput screens of the Prestwick (compounds P1-P12) and Lopac (compounds L1-L12) chemical libraries. Dark blue columns represent FA values for a solution containing FITC-E2A and KIX; light blue columns represent the FA value after the addition of compound. Note that most compounds failed to cause a reproducible decrease in FA when re-tested in this “low-throughput” manner. Of the 43 initial hits, 10 compounds (P1, P2, P9, L1, L2, L3, C1, C2, C9, C15) caused a reproducible decrease in FA.

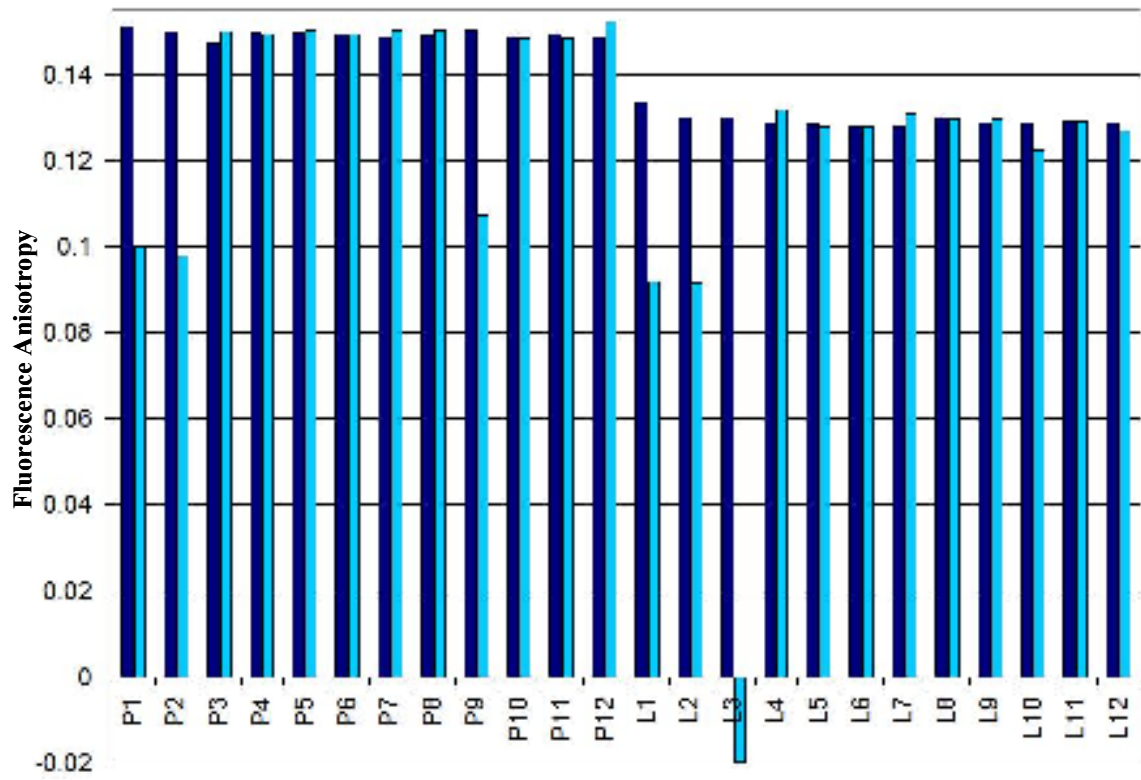
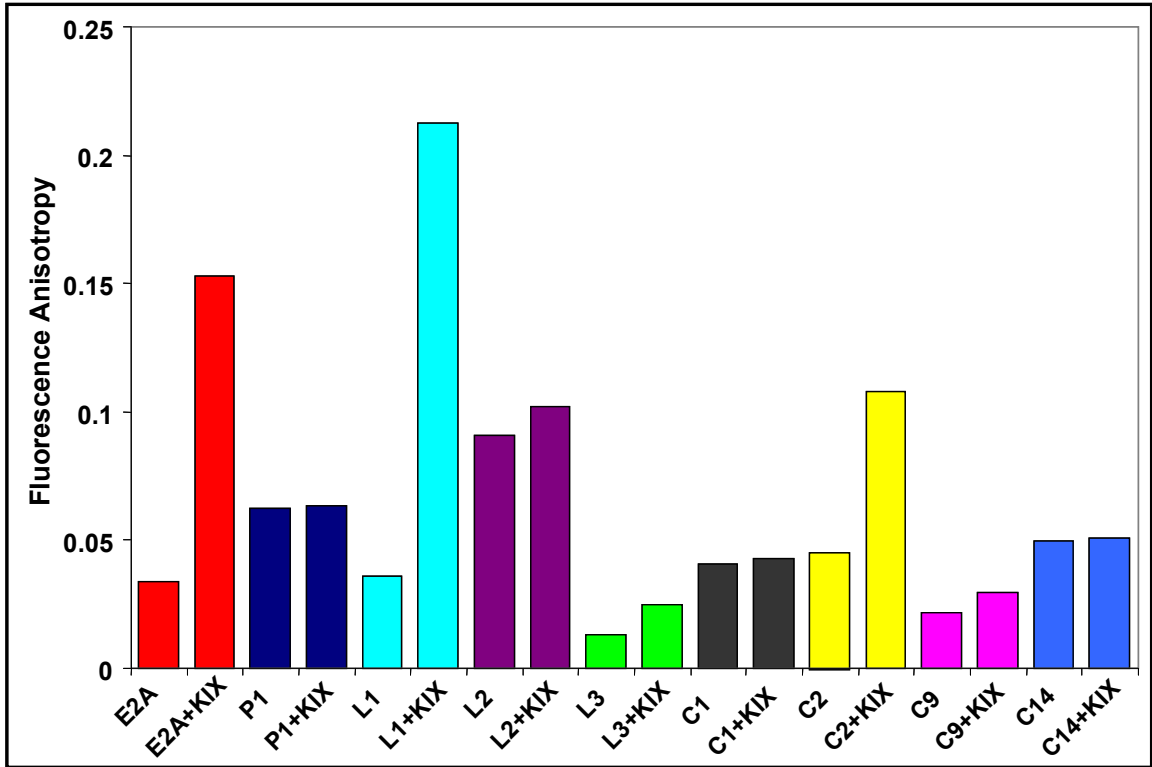


Figure 3-6 FA of fluorescent compounds exploited to demonstrate KIX interactions

Each sample is given a different colour, and for each one the bar on the left indicates FA of 1 μ M of sample alone, and on the right indicates FA after KIX was added. As a reference to indicate true KIX interaction, FITC-E2A alone and after KIX was added are shown on the left side in red. Compounds L1 (light blue) and C2 (yellow) both demonstrated possible true interactions with KIX. All other fluorescent compounds were excluded from further analysis due to their interference with the FITC-E2A fluorescent signal.



interaction were discarded. Since the small molecules were added in concentrations far above that of FITC-E2A, the FA values were largely based on the fluorescence of the small molecules. FA of the small molecules is extremely low, and mimics the FA of FITC-E2A alone, therefore the fluorescent small molecules appear to inhibit the FITC-E2A:KIX interaction. Although the FA of compound L3 appears to increase modestly upon addition of KIX in figure 3.5, the intrinsic fluorescent signal of this compound was substantially greater than FITC-E2A, therefore it was ruled out as a false positive as it interfered substantially with the FITC-E2A fluorescent signal.

3.5.2 FA of fluorescent compounds exploited to demonstrate KIX interactions

Compound L1 is a fluorescent compound with an FA value of approximately 0.036 when alone in buffer. This value increased six-fold upon the addition of KIX (**Figure 3-6**), which implied a strong interaction with the KIX domain. Therefore, KIX was titrated into a solution containing 1 μM L1 and the effects on FA, as measured using the intrinsic fluorescence signal from L1, were monitored to establish a binding curve. The addition of KIX to concentrations ranging from 0 μM to approximately 35 μM caused an abrupt increase in FA which steadily increased and eventually reached a plateau (**Figure 3-7 A**). The titration curve looked similar to that of E2A:KIX, and was performed in duplicate to ensure the results were reproducible. When the data were fit to a hyperbolic equation, we obtained a K_D of approximately 3 μM , indicating relatively tight binding to KIX. NMR spectroscopy and isothermal titration calorimetry (ITC) were used to analyze interactions between L1, KIX and GST-E2A. In these experiments, L1 formed a precipitate upon addition to solutions containing KIX or GST-E2A. Furthermore, it showed no significant cytotoxicity on myeloid cells immortalized by E2A-PBX1 or in human leukemia-derived cell lines when tested with the MTT assay (data not shown). Due to the solubility issues and relative lack of cytotoxic effects, further testing of compound L1 was not pursued.

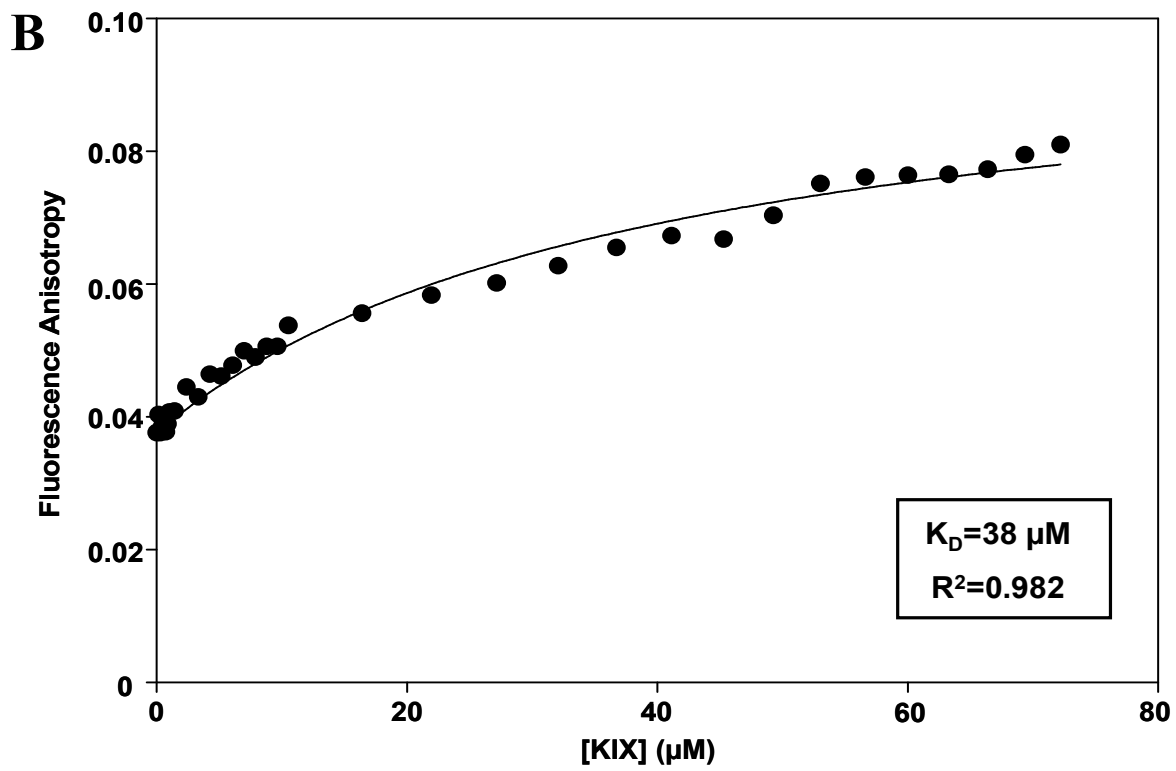
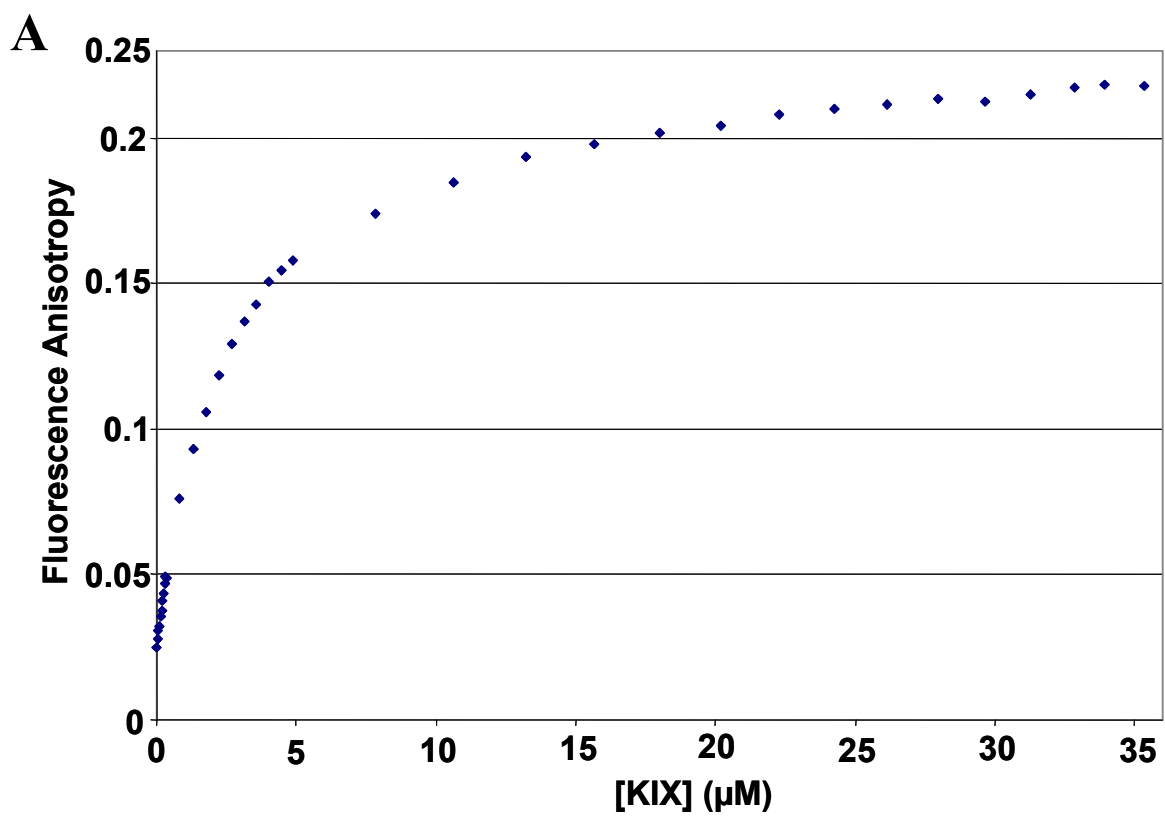
Figure 3-7 FA of fluorescent compounds exploited to demonstrate KIX interactions

(A) A titration of compound L1 with KIX shows an interaction with a 3 μM K_D

1 μM of compound L1 alone had an FA value of 0.036 which increased steadily upon addition of recombinant KIX domain, from 0 μM to 35 μM . The FA values reached a plateau after approximately 15 μM was added, and the data were fit to an equation which showed a binding constant of 3 μM .

(B) A titration of compound C2 with KIX shows an interaction with a 38 μM K_D

1 μM of compound C2 alone had an FA value of 0.03, which increased slowly upon addition of recombinant KIX domain, with a slope indicative of a weaker interaction. The FA values did not plateau until 70 μM of KIX was added to compound C2, and the K_D of the interaction was determined to be 38 μM .



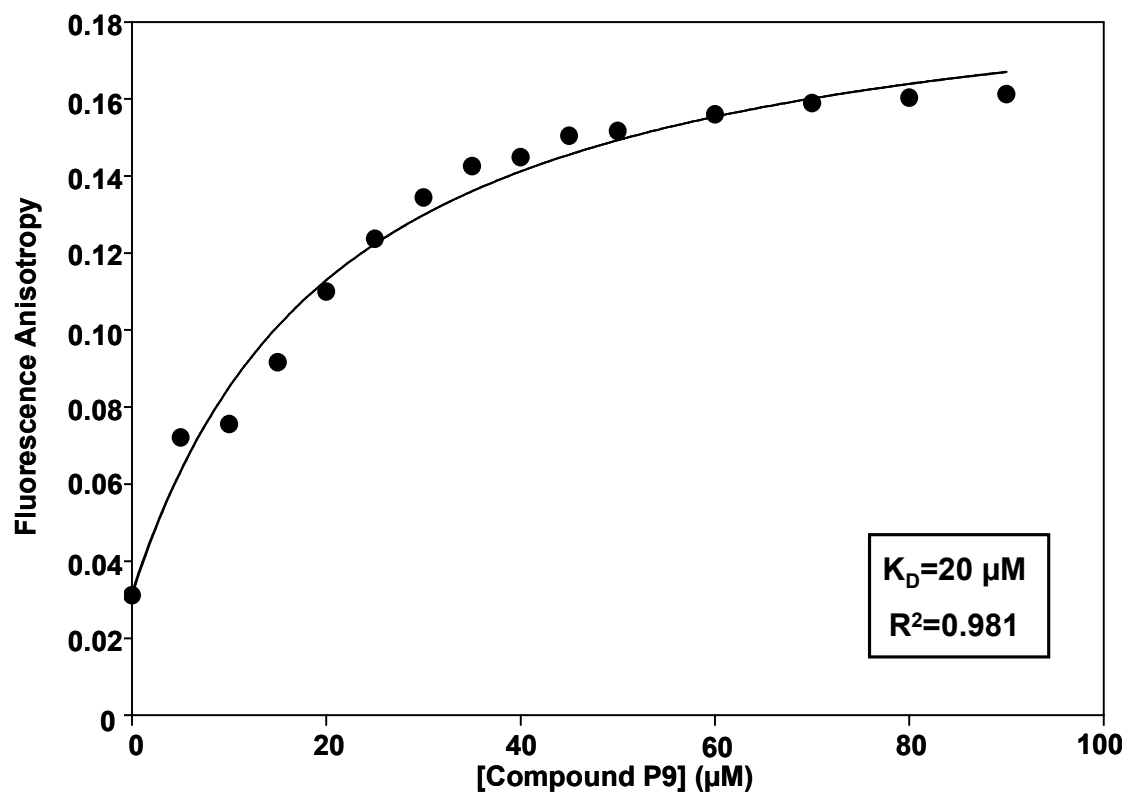
The intrinsically fluorescent compound C2 from the Chembridge library also demonstrated a potential KIX interaction. A wave scan was performed to determine that the maximal excitation and emission wavelengths of the compound were 506 nm and 536 nm, respectively. Compound C2 alone had an FA value of approximately 0.03, a value expected for a small unbound molecule. When recombinant KIX was titrated into the compound the FA values increased but with a gradually increasing slope, indicative of a modest affinity (**Figure 3-7 B**). When the data were fit to a hyperbolic equation, a K_D of 38 μM was obtained. This fluorescent compound was pursued for further studies since it causes a decrease in FA of the E2A:KIX interaction, and although it is intrinsically fluorescent, interacts with KIX with a 38 μM K_D .

3.5.3 FA of FITC-E2A exploited to detect an interaction with compound P9

One of the positive hits from the Prestwick library, compound P9, did not demonstrate endogenous fluorescence at any wavelength. Using FA as determined using the fluorescent signal from FITC-E2A, we investigated a potential interaction between FITC-E2A and compound P9. The addition of compound P9 to FITC-E2A resulted in more than a two-fold increase in FA, from approximately 0.03 to 0.08. This increase indicated that there was an interaction occurring between compound P9 and the E2A peptide. A full titration was performed with increasing concentrations of compound P9 titrated into 1 μM FITC-E2A, and a K_D was obtained of 20 μM (**Figure 3-8**). The fluorescently labeled E2A peptide has a molecular weight of approximately 1.9 kDa, which is only three times larger than compound P9, perhaps accounting for the ability of compound P9 to result in a substantial increase in FA upon interaction with FITC-E2A. Compound P9 was characterized further based on the following considerations: 1) it inhibits E2A:KIX binding, as indicated by FA, and is not intrinsically fluorescent; 2) it binds to the E2A peptide with reasonable affinity, suggesting that disruption of the E2A:KIX complex is mediated by primary, direct binding to the E2A component.

Figure 3-8 Compound P9 binds to FITC-E2A with a K_D of 20 μM

The non-fluorescent compound P9 from the Prestwick library was titrated into a 1 μM sample of FITC-E2A up to a concentration of approximately 90 μM . Compound P9 caused an increase in FA from 0.03 to almost 0.16, resulting in a plateau after approximately 40-50 μM was added. Compound P9 interacts with E2A with a K_D of approximately 20 μM .



3.6 Biochemical and biophysical characterization of validated hits

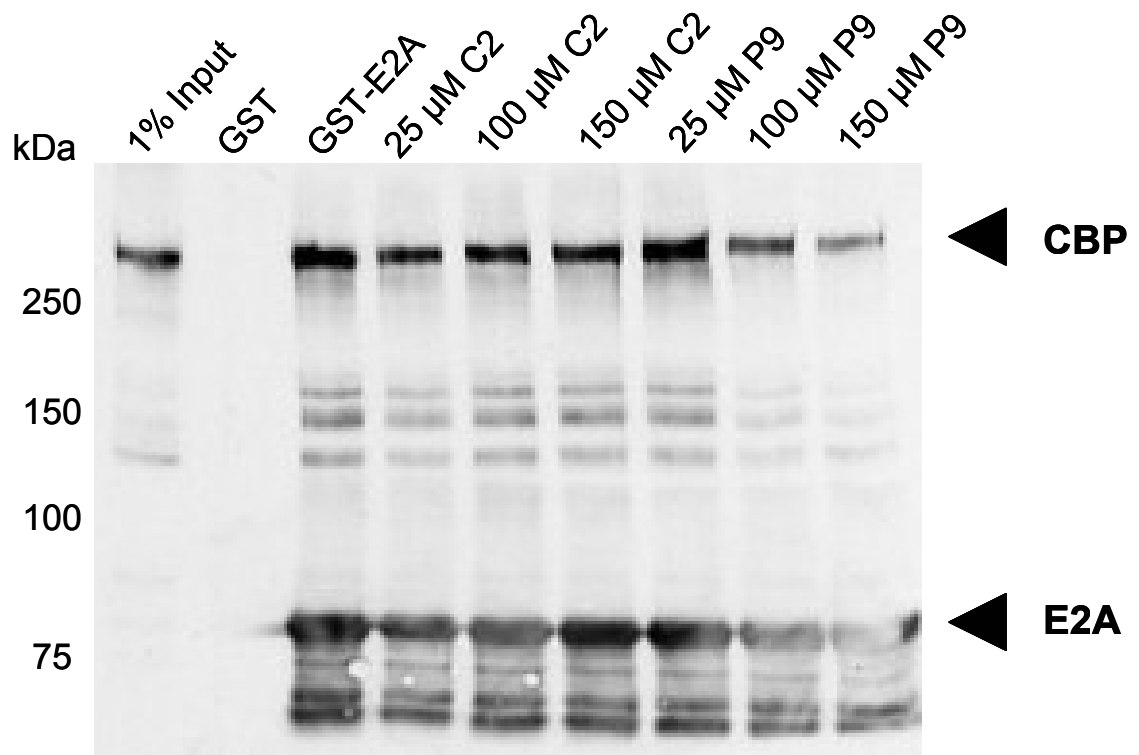
Subsequent to validating positive hits from the high throughput screening in causing a reproducible decrease in the FA of FITC-E2A:KIX and determining possible interactions between the small molecules and KIX or E2A using FA, the remaining compounds, P9 and C2, were then characterized using biochemical assays. Although the validated hits demonstrated inhibition of the interaction between a small synthetic peptide, FITC-E2A, and the isolated, recombinant KIX domain in the FA assay, the next step was to determine if these compounds could inhibit the interaction between larger fragments of these proteins, such as a longer portion of E2A and full length CBP. This is an important question, as interactions between the larger proteins are more biologically relevant. Furthermore, there are many additional residues within the full-length CBP protein and E2A-PBX1 fusion protein which offer important potential contact points *in vivo*. Small molecule inhibitors are likely to be effective only if they are capable of inhibiting these full length interactions found within ALL patients.

3.6.1 Pull down assays

Pull down assays were performed using GST-E2A (aa 1-483) to pull down full length CBP from RCH ACV nuclear extracts. Varying concentrations of compounds P9 and C2 were tested for their ability to inhibit this interaction between GST-E2A and full length CBP. Compound P9 caused a reduction in the amount of CBP pulled down by GST-E2A, at concentrations as low as 100 μ M (**Figure 3-9**). The ability of compound P9 to inhibit binding between the full-length proteins was dose dependent and corroborates the previous FA results. Compound C2 was unable to inhibit the interaction between E2A and CBP in this assay at concentrations up to 150 μ M. Therefore, further studies of this compound were not pursued.

Figure 3-9 Compound P9 inhibits full length E2A:CBP in a pull down assay

Compound P9, and not C2, demonstrates inhibition of the full length E2A:CBP interaction in a pull down assay. GST-E2A (aa 1-483) was used to pull down full length CBP from RCH ACV nuclear extracts; compounds C2 and P9 were added to the GST-E2A in the indicated concentrations simultaneous to the addition of nuclear extract, to inhibit the CBP interaction. Compound P9 was able to inhibit this interaction at concentrations above 100 μ M in a dose dependent manner.

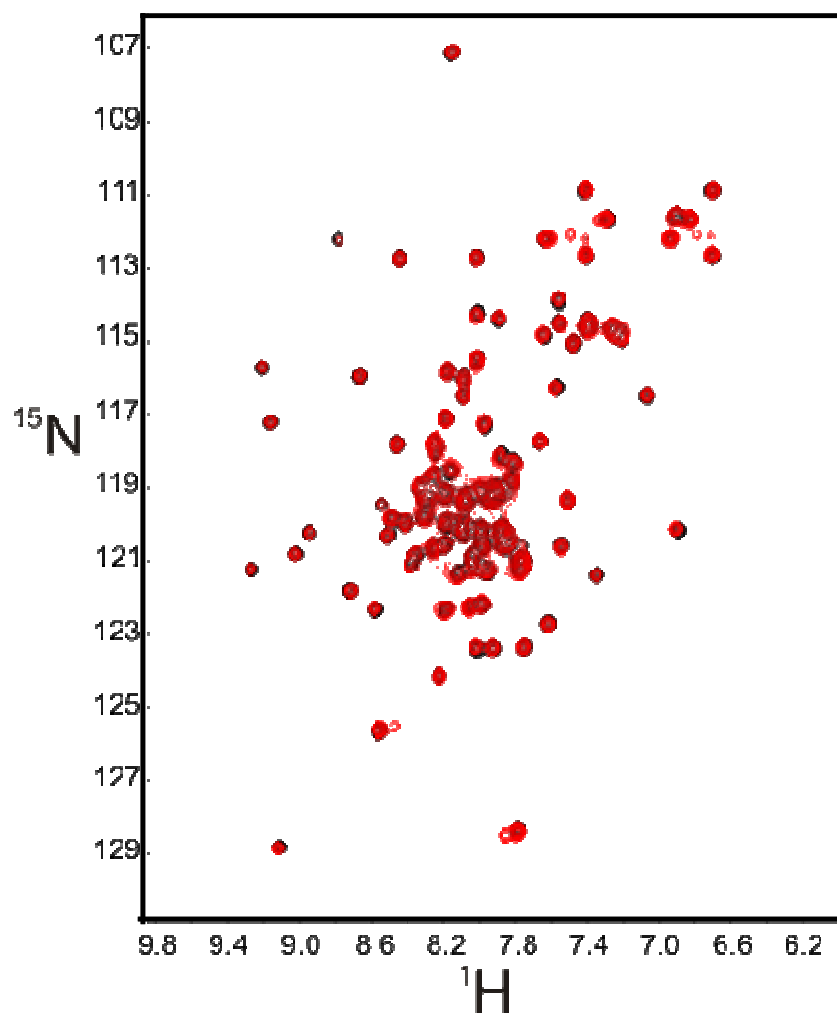


3.6.2 NMR spectroscopy

The KIX domain has previously been solved by NMR spectroscopy, formed by three alpha helices which are joined by two loop structures. Many interacting partners of KIX have been mapped to the KIX domain, but only recently in our lab has the E2A peptide binding site been mapped to the KIX domain (Chris Denis, unpublished data). Using isotopically $^{13}\text{C}/^{15}\text{N}$ labeled recombinant KIX, $^1\text{H}-^{15}\text{N}$ heteronuclear single quantum coherence (HSQC) experiments can be used to determine the chemical shifts of each of the amino acids within the KIX domain. Each nucleus within the protein is surrounded by a distinct chemical environment which allows it to be recognized by a distinct chemical shift. Obtaining the chemical shift data from each of the amino acids allows the domains to be mapped, forming a 3D KIX structure. Within the mapped $^{13}\text{C}/^{15}\text{N}$ KIX domain, the amino acids which shift upon interacting with E2A have also been determined, using the same principles. These amino acids which form contacts with E2A can be compared to the amino acids which shift within KIX upon interaction with small molecules, in order to compare binding sites. This serves as useful information when detecting a possible interaction between candidate hits and the KIX domain, as we are looking for shifts in the same amino acids affected by the E2A peptide. When compound P9 was added to $^{13}\text{C}/^{15}\text{N}$ -labeled KIX, the resonances representing the E2A-interacting amino acids did not display any significant chemical shift changes. Rather, there was almost perfect overlap between the KIX spectrum and the KIX spectrum after P9 was added (**Figure 3-10**). The fact that compound P9 causes a reproducible decrease in FA when added to FITC-E2A:KIX and does not appear to interact with the KIX domain when tested using NMR spectroscopy corroborates the previous FA result of an interaction between compound P9 and the E2A peptide.

Figure 3-10 Compound P9 does not interact with the KIX domain by NMR spectroscopy

Each point in the plot represents an amino acid, with black points representing the amino acid peaks of $^{13}\text{C}/^{15}\text{N}$ KIX alone and red points representing $^{13}\text{C}/^{15}\text{N}$ KIX after addition of compound P9. Since there is almost perfect overlap of the amino acid peaks before and after the addition of compound P9, there is no indication of an interaction between KIX and compound P9. If compound P9 interacted with the KIX domain, it would be expected that the amino acids within KIX which contribute to the interaction would shift.



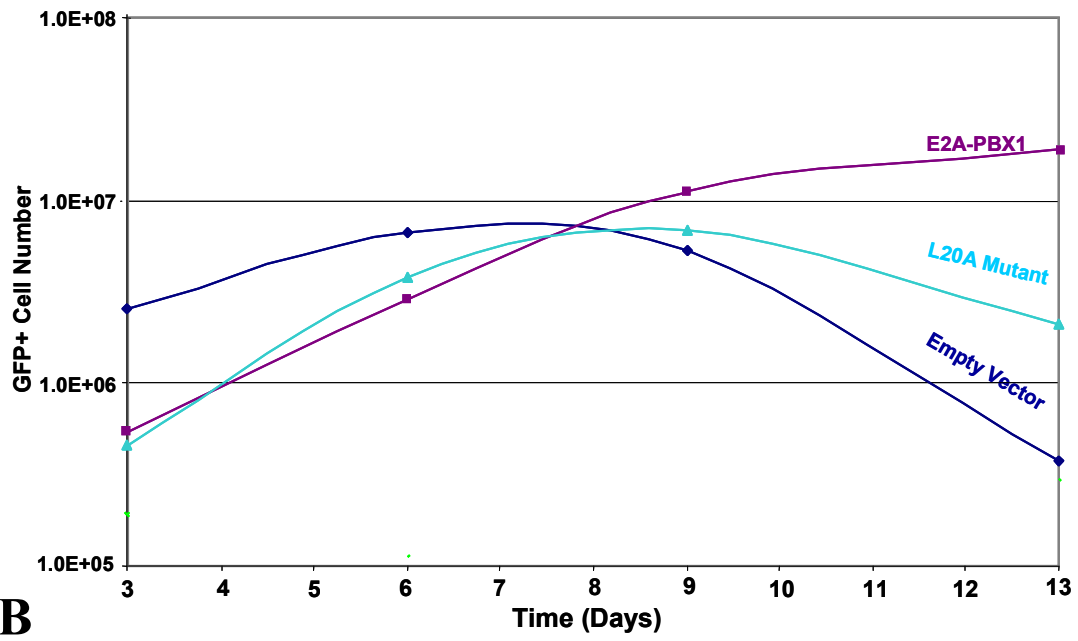
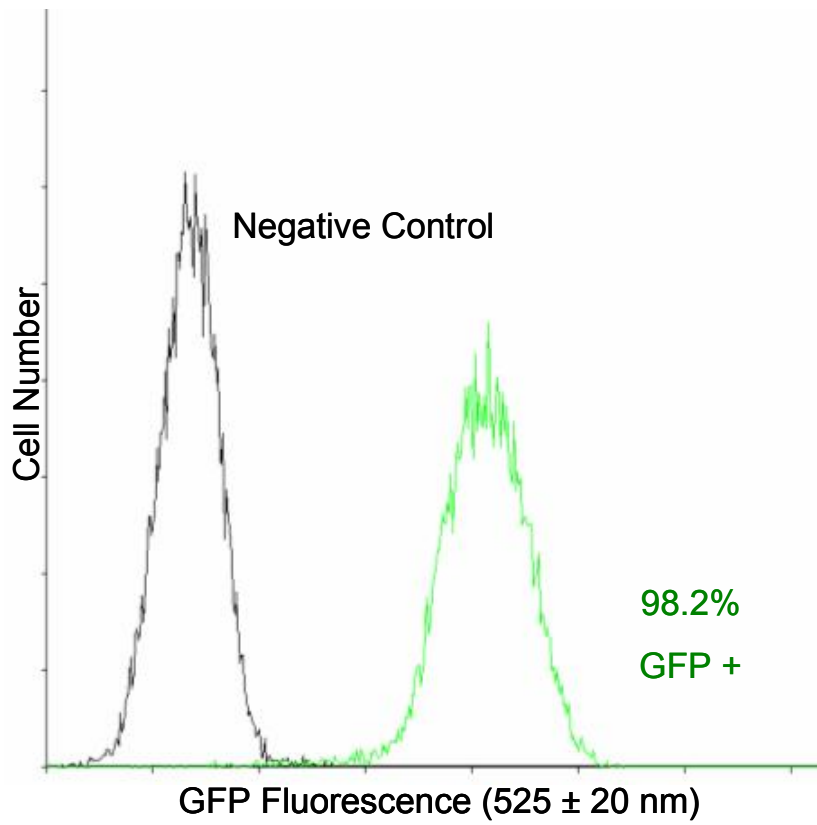
3.7 Biological characterization of positive hits

3.7.1 E2A-PBX1-infected bone marrow cells

The next step was to determine the cytotoxic effects of the small molecules on cells which are dependent on E2A-PBX1 for survival in order to investigate the idea that the mechanism of action of the small molecules is through inhibitory effects on the E2A-PBX1:CBP interaction. Although leukemic cell lines that express endogenous E2A-PBX1 have been established from patients, these have been propagated in cell culture for years. To serve as a fresh cell line, solely dependent on E2A-PBX1 for survival, primary murine bone marrow cells were infected with a retrovirus that conferred simultaneous expression of E2A-PBX1 and green fluorescent protein (GFP). The GFP allows for the enumeration of E2A-PBX1-expressing cells over time using flow cytometry. The E2A-PBX1 infected cells grew similar to control cells, infected with an empty MIEV retrovirus, until 8 days post-infection. At this point, cells infected with empty vector, or the L²⁰A mutant of E2A-PBX1, differentiated into short-lived granulocytes or adherent macrophages and no longer propagated in culture. In contrast, cells infected with E2A-PBX1 became immortalized and were able to proliferate in media containing GM-CSF for months (**Figure 3-11 A**). After day 8, the portion of cells expressing GFP, and consequently E2A-PBX1, exceeded 98% (**Figure 3-11 B**). The immunophenotype of these cells established that they represent a population of immortalized myeloid progenitors, expressing myeloid cell surface markers. This E2A-PBX1-immortalized myeloid (EPM) cell line is advantageous for the purpose of evaluating the potential effects of small molecules on E2A-PBX1 function because these cells are proliferating in culture solely due to the biological effects of recombinant E2A-PBX1.

Figure 3-11 E2A-PBX1 infected bone marrow cells become immortalized myeloid progenitors

- (A) Cells infected with a MIEV retrovirus, conferring expression of GFP and E2A-PBX1 in the same coding sequence, initially grow comparably to cells infected with an empty MIEV retrovirus and the L²⁰A mutant of E2A-PBX1. After day 8, E2A-PBX1-infected cells continue to proliferate, whereas the negative control cells (MIEV and L²⁰A mutant) begin to die. E2A-PBX1-infected bone marrow cells become immortalized myeloid progenitors and proliferate in GM-CSF media indefinitely.
- (B) After day 8 over 98% of E2A-PBX1-infected cells express GFP, and consequently E2A-PBX1. This proportion of cells remains constant over time, ensuring that E2A-PBX1 expression is correlated with immortalization.

A**B**

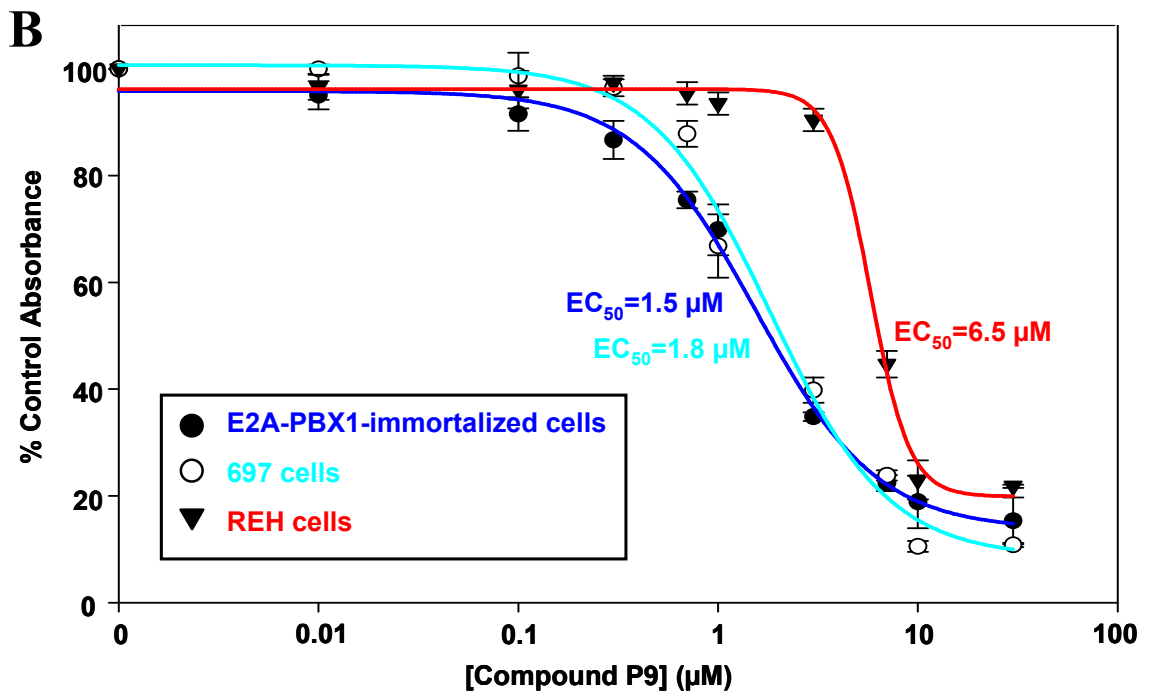
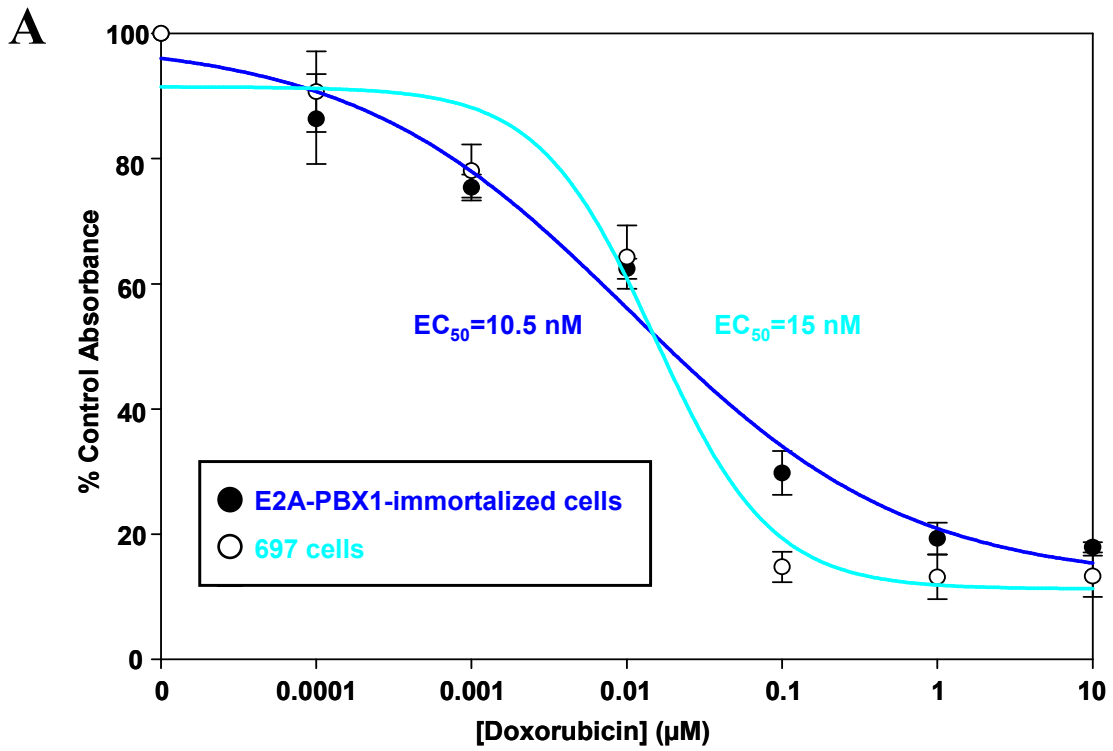
3.7.2 MTT cytotoxicity assay validation

The MTT assay is a colorimetric assay used to detect the cytotoxicity of drugs or small molecules. Cells are treated with the drug of interest, and the ability of the cells to reduce the yellow MTT compound to blue formazan, a measure of the cellular viability and enzymatic activity, is detected after 72 hours. This assay has proven useful in many different cell lines; however it has never been used with the EPM cells which depend solely on E2A-PBX1 for survival. In order to ensure that this assay can properly detect the cytotoxicity of a drug on cell lines expressing E2A-PBX1, we used the known chemotherapeutic drug doxorubicin.

Doxorubicin is an anthracycline used in remission-induction therapy for patients with ALL. Both EPM cells and the leukemic cell line 697, which also expresses E2A-PBX1, were tested with doxorubicin. This preliminary experiment was performed to ensure that the MTT assay detects cytotoxicity in both cell lines with known chemotherapeutic drugs. This served as a source of comparison for the efficacy of the candidate small molecules. Effective drug concentrations are measured with EC_{50} values, which is a measure of the concentration of the compound demonstrating toxicity in half of the cells. At increasing concentration, doxorubicin had a similar toxic effect on both of the E2A-PBX1-expressing cell lines. The EC_{50} of doxorubicin on the EPM cells was slightly lower than that of the 697 cells; 10.5 nM and 15 nM, respectively (**Figure 3-12 A**). This experiment validated the use of the MTT assay in properly detecting drug cytotoxicity, as well as offered a source of comparison for EC_{50} values of a known chemotherapeutic drug. Although the EC_{50} of doxorubicin is cell line independent, it is generally in the low nanomolar range.

Figure 3-12 Compound P9 is slightly more toxic on cell lines which express E2A-PBX1

- (A) The cytotoxicity of doxorubicin, a drug used to treat ALL, was tested on E2A-PBX1-expressing cell lines to validate the MTT assay and determine the EC_{50} of a well characterized drug. Doxorubicin showed cytotoxic effects on both cell lines, E2A-PBX1-immortalized bone marrow (EPM) cells and the leukemic 697 cell line. The EC_{50} values were 10.5 nM for EPM cells and 15 nM for 697 cells.
- (B) Compound P9 demonstrated slightly higher cytotoxicity on cells which express E2A-PBX1 (697 and EPM cells) as compared to leukemic cells lacking E2A-PBX1 expression (REH cells). The EC_{50} values of compound P9 on EPM and 697 cells were 1.5 and 1.8 μ M, respectively, and for REH cells was 6.5 μ M. This experiment was performed in duplicate to ensure the reproducibility of results.



3.7.3 MTT cytotoxicity assay for validated hits

The MTT assay was subsequently used to evaluate compound P9 for toxicity and selectivity for cells which express E2A-PBX1. DMSO was present as a vehicle in the high throughput screening; the concentration of DMSO was kept constant at a concentration of 0.2% in all samples. Compound P9 demonstrated a modestly higher cytotoxicity on EPM and 697 cells, which express E2A-PBX1, relative to REH cells, which do not express E2A-PBX1. Data was fit to a sigmoidal dose-response curve, and EC₅₀ values were 1.5 μ M, 1.8 μ M and 6.5 μ M for E2A-PBX1-immortalized cells, 697 cells and REH cells, respectively (**Figure 3-12 B**). The existence of an apparent therapeutic window, albeit a relatively narrow one, is encouraging in that it suggests that compound P9 may be at least somewhat selective for neoplastic cells that express E2A-PBX1.

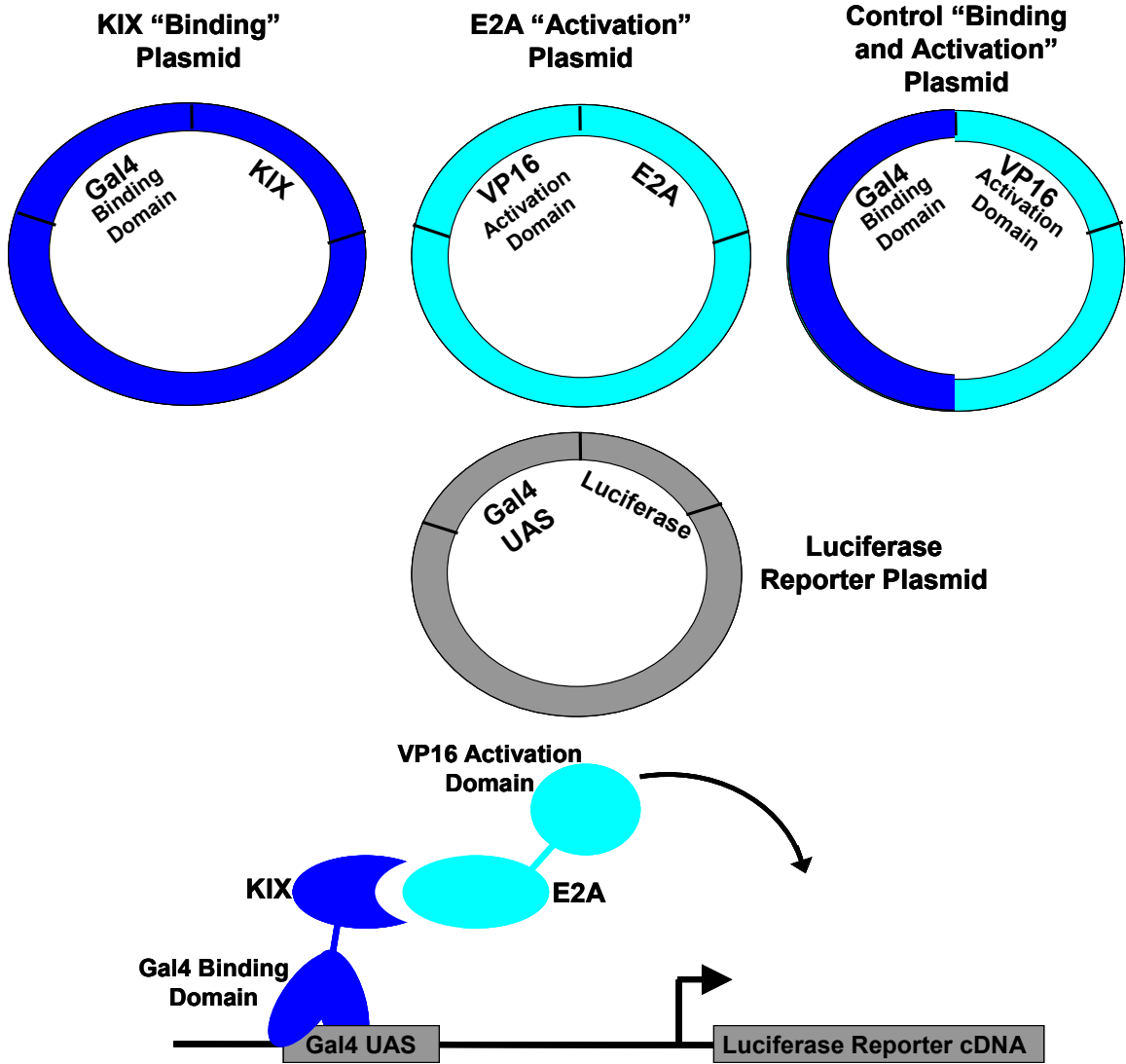
3.7.4 Mammalian two-hybrid assay

The next step in the biological validation of the hits was to perform a dual-luciferase mammalian two-hybrid assay. This assay can be used to detect an interaction between two proteins, when they are fused to either a GAL4 binding domain or a VP16 activation domain. Expression of a luciferase reporter gene, detected by a luminometer, is controlled by a promoter containing GAL4 binding domains and occurs only if the GAL4 and VP16 domains are brought into close proximity by the interaction of the proteins of interest (**Figure 3-13 A**). A GAL4-KIX fusion protein and VP16-E2A fusion protein were expressed in SV293T cells and the expression of luciferase, consequent to the KIX:E2A interaction, was detected. If the small molecules were able to inhibit the E2A:KIX interaction, then the GAL4 and VP16 domains would no longer be in close proximity to each other, and the luciferase expression would be expected to decrease. This assay is a biological read out of the E2A:KIX interaction in living cells, and is also an indicator of whether or not the small molecules are able to enter living cells to result in the disruption of the

Figure 3-13 Mammalian two-hybrid assay

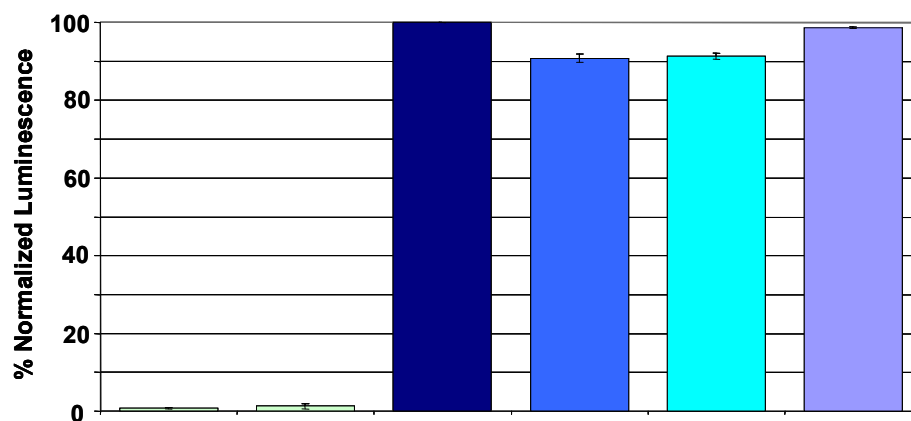
- (A) The KIX “binding” plasmid and E2A “activation” plasmid confer the expression of KIX fused to the GAL4 binding domain, and E2A fused to the VP16 activation domain. In order for the luciferase reporter gene to be expressed, the GAL4 binding domain must interact with GAL4 upstream activating sequences (UAS) and the VP16 domain must be in close proximity to activate transcription of luciferase, which occurs if the KIX and E2A domains interact. The control “binding and activation” plasmid confers expression of the GAL4 binding domain fused to the VP16 activation domain, which will constitutively activate luciferase expression from the reporter plasmid.
- (B) Control normalized luciferase expression, caused by the GAL4-KIX:VP16-E2A interaction, was set to 100%. Compound P9 resulted in a decrease to 90% of control luciferase expression at both 5 μ M and 10 μ M, implying inhibition of the E2A:KIX interaction. However, the decrease in luciferase expression at both concentration points was not significant ($p>0.37$).
- (C) Negative control normalized luciferase expression, caused by the GAL4-VP16 fusion protein, was set to 100%. Compound P9 resulted in a slight decrease to 96% control luciferase expression at 10 μ M. Compound P9 should have no effect on the expression of luciferase caused by the negative control fusion protein, since it is a fusion of the binding and activation domains which should activate the reporter gene irrespective of KIX interactions. Both experiments were performed in duplicate to ensure reproducibility of results.

A

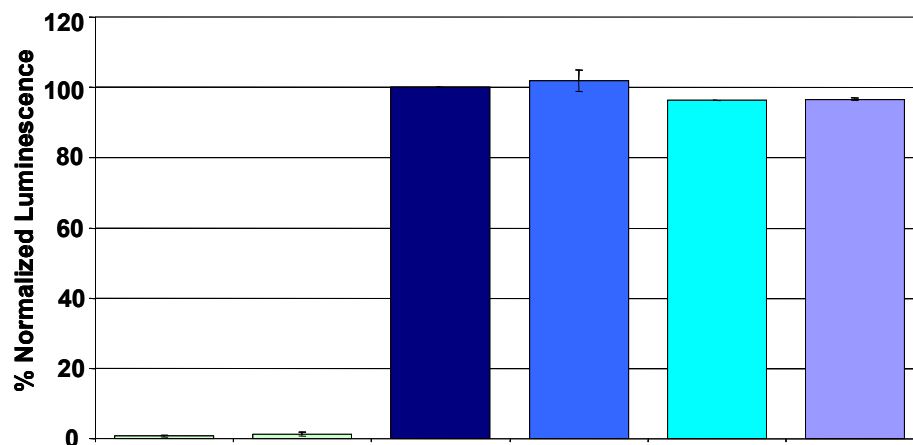


B

VP16	-	+	-	-	-	-
VP16-E2A	+	-	+	+	+	-
GAL4	+	-	-	-	-	-
GAL4-KIX	-	+	+	+	+	-
Compound P9	-	-	-	5 μM	10 μM	-
0.2% DMSO	+	+	+	+	+	+

**C**

VP16	-	+	-	-	-	-
VP16-E2A	+	-	-	-	-	-
GAL4	+	-	-	-	-	-
GAL4-KIX	-	+	-	-	-	-
VP16-GAL4	-	-	+	+	+	-
Compound P9	-	-	-	5 μM	10 μM	-
0.2% DMSO	+	+	+	+	+	+



interaction. As a control for the effects of the drugs, to ensure that any effects they were causing were due to inhibition of the KIX:E2A interaction, a GAL4-VP16 fusion protein was tested against the same concentrations of compounds. This construct constitutively induces luciferase expression thanks to the presence of the VP16 activation domain from Herpes simplex. Since transcriptional induction by VP16 is not known to rely on KIX binding, compounds that disrupt E2A:KIX binding would be expected to exert differential deleterious effects on luciferase induction consequent to co-transfected VP16-E2A and GAL4-KIX relative to that induced by GAL4-VP16.

For this assay, luciferase associated with co-transfection of VP16-E2A and GAL4-KIX is set to 100% and the results obtained on the addition of compound P9 are normalized to this value. Addition of compound P9 caused a slight reduction in luciferase expression to 90% of control at either 5 μ M or 10 μ M concentrations (**Figure 3-13 B**). However, compound P9 at 10 μ M also showed a slight 96% reduction in the GAL4-VP16 control luciferase expression (**Figure 3-13 C**). DMSO was present in the high throughput screening as a vehicle for the compounds, and in this assay the concentration of DMSO was reduced and kept constant at a final concentration of 0.2% of the sample volume for each condition. DMSO did not have a significant effect on control VP16-E2A:GAL4-KIX or GAL4-VP16 luciferase expression. This experiment was performed in duplicate and the effect of compound P9 on the luciferase expression was not significant; 5 μ M of compound P9 reduced luciferase expression to 90% control ($p=0.37$), and 10 μ M of compound P9 reduced luciferase expression to 90% control ($p=0.49$).

Chapter 4

Discussion

4.1 High throughput screening and FA-based assays

The interaction between the chimeric protein E2A-PBX1 and CBP has been implicated in leukemogenesis in a subset of ALL cases. This oncogenic interaction is dependent on a single leucine residue within a conserved LXXLL motif in E2A-PBX1, which interacts with the KIX domain of CBP. Engineering a point mutation, L²⁰A, within E2A-PBX1 inhibits the interaction *in vitro* and prevents the *in vivo* E2A-PBX1-mediated leukemia induction in mouse models. We hypothesized that this oncogenic interaction is susceptible to small molecule inhibition. Furthermore, I investigated this interaction with an FA-based assay and found a small molecule, denoted P9, which inhibits the E2A-PBX1:CBP interaction and has cytotoxic effects on E2A-PBX1-immortalized myeloid cells. This inhibitor represents a novel lead compound for the development of an improved treatment for this subset of ALL cases.

FA-based assays are the most popular method for high throughput screening, applicable to protein-protein, protein-DNA and receptor-ligand interactions. Recently, Mao *et al.* used an FA-based binding assay to find an inhibitor of a nuclear receptor-DNA interaction⁶⁸, and Moerke *et al.* used this technique to find an inhibitor of a protein-protein interaction⁶⁹. Our FA-based assay showed a relatively high affinity for the interaction between FITC-E2A and KIX with a K_D of 2 μ M. The assay proved to be reproducible, robust, and could properly detect inhibition of the interaction. Many different buffer conditions were tested to obtain this K_D value, including various pH buffers, pH values and salt concentrations. The conditions that produced the tightest interaction were 20 mM MES pH 6.0 with no additional salt. The interaction got weaker as the salt concentration and pH increased. Although a buffer containing no salt at pH 6.0 is not representative of physiological conditions, it is essential to have a relatively strong interaction for

the assay to be useful for high throughput screening. If the K_D increased substantially above 2 μM , the stringency of the assay would decrease, potentially leading to an unacceptably high number of false-positive hits. Our buffer conditions were similar to those previously used in NMR studies of the interaction between KIX and E2A. We were thus able to justify the use of relatively non-physiological buffer conditions.

The FITC molecule conjugated to the E2A peptide has optimal fluorescence at a pH range of 9-9.5. Nonetheless, the FITC-E2A peptide gave an adequate signal at pH 6.0. Many of the small molecules had higher intrinsic fluorescence than FITC-E2A, perhaps in part due to the pH dependence of the FITC tag. The use of pH-independent fluorescent tags has been investigated. BODIPY, non-ionizable fluorescent dyes, can be conjugated to peptides and allows for a strong pH independent fluorescent signal. Using a BODIPY tag could eliminate the pH dependence issues encountered with the FITC tag⁷⁰.

We successfully screened three small molecule libraries. Of the 12400 compounds that were screened, 43 emerged as positive hits from the initial screens. Of these initial positive hits, 10 were validated in their ability to cause a reproducible decrease in FA. After fluorescent compounds that showed no interaction with KIX were removed, 3 compounds remained: compounds P9, L1, and C2 (**Table 3.1**). The substantial number of false positives in our study was due to the fact that 9 of the 10 compounds that presented as positive hits from the screens and were validated in causing a reproducible decrease in FA were intrinsically fluorescent at the visible wavelengths used for the assay. The FA values were calculated based on the assumption that the emitted fluorescence was from the FITC-E2A peptide. However, intrinsic fluorescence associated with the small molecules was at levels higher than that of FITC-E2A when tested in equimolar concentrations, and they were added at concentrations eight times greater than FITC-E2A. They were therefore likely to have produced a false FA result, since the

measured FA value would have been calculated based on the sum total fluorescence from FITC-E2A and the small molecule, and the fluorescence of the small molecule was far greater than that of FITC-E2A. Since the former is expected to tumble more rapidly due to its small size, the measured FA is likely to have been spuriously low, thus mimicking the effect of disrupting E2A-KIX binding. Subsequently, intrinsically fluorescent compounds were titrated against KIX (in the absence of FITC-E2A) and FA was calculated based on the fluorescence from the small molecule. Compounds were excluded from further study if they did not demonstrate an interaction in this experiment. Mao *et al.* performed similar FA-based high throughput screening and were able to avoid this problem of confounding fluorescence, by detecting the basic fluorescence of the compounds directly in the plate⁶⁸. Of their 262 initial hits, only 56 remained (21%) after those that were fluorescent were removed. Consistent with these findings, 3 of 19 of our initial hits (16%) remained after a similar elimination process. Performing these fluorescence-based analyses directly on the plate would be an efficient step to reduce the high rate of intrinsically fluorescent hits.

Improvements for future development of an assay based on FA could include the use of a fluorescent tag associated with excitation and emission wavelengths higher or lower than those of FITC (492 and 523 nm, respectively), as this is where a relatively large number of small molecules from the libraries demonstrate fluorescence⁷¹. The use of other fluorescent tags for the purposes of FA-based binding assays has been successful, such as tags in the red visible wavelengths⁷⁰. BODIPY and Texas Red are examples of such fluorescent tags, with higher excitation and emission wavelengths of 590 nm and 615 nm, respectively. Small molecules would be less likely to interfere with the FA signal of E2A conjugated to one of these fluorescent tags. Consequently, fewer positive hits would be false positives, and there would be no interference of the fluorescent signal when the small molecules were being validated with FA.

4.2 Inhibiting protein-protein interactions involving the KIX domain

Compound P9 was the only non-fluorescent, validated hit that inhibited FITC-E2A:KIX binding in the FA assay. Furthermore, P9 inhibited binding of E2A (aa 1-483) to full-length CBP in a pull-down assay with the highest efficacy and produced cytotoxic effects in cultured, E2A-PBX1-expressing cells. This compound demonstrated an interaction with the E2A peptide with a K_D of 20 μ M, and failed to demonstrate a KIX interaction by NMR spectroscopy. It is important to determine the specificity of compound P9 to the E2A domain. Other E proteins contain homologous sequences, and many of the proteins that interact with the KIX domain, such as c-Jun, c-Myb and MLL, share the highly conserved LXXLL motif found within E2A. The specificity of compound P9 for E2A can be determined by evaluating the binding of P9 to peptides that represent E proteins, c-Jun, c-Myb or MLL. Alanine scanning of the E2A peptide could be employed to determine important residues within E2A that contribute to the interaction with P9. It is also possible to use isotopically labeled E2A to solve the E2A structure and map the binding site of compound P9 using NMR spectroscopy. Although this would require more time, it would also aid in the development of a more specific compound to inhibit this E2A-PBX1:CBP interaction.

CBP is a large transcriptional co-activator that is commonly recruited to DNA-binding transcription factor complexes. Often, the recruitment is mediated by the KIX domain of CBP. An inhibitor that interacts with the E2A peptide could be superior to one that interacted with the KIX domain, as it would be difficult to find an inhibitor specific to the E2A binding site if the inhibitor bound primarily to KIX. An inhibitor that interacts with E2A, such as P9, would likely be a more specific inhibitor of the E2A:KIX interaction, considering the large number of KIX interacting partners. Although E2A-PBX1 binds to its own site on the KIX domain, some of the residues of the E2A binding site overlap with other binding sites. It would therefore be expected

that small molecule inhibitors of the KIX:E2A interaction that interact with the KIX domain could inhibit more than one KIX interaction. Best *et al.* reported a small molecule which interacts with the KIX domain to inhibit CREB binding, with a K_D of 90 μM ⁶⁷. Buhrlage *et al* also reported a small molecule which interacts with the KIX domain at the MLL/Jun binding site with a K_D of 38 μM ⁷². In the current study, two compounds were found to interact with KIX; compounds L1 and C2 both interacted with low K_D values of 3 μM and 38 μM , respectively. Compound L1 was discarded for its lack of solubility and cytotoxic effects, and compound C2 was discarded as a false positive for failure to inhibit the full-length interaction in a pull down assay. However, comparing their interaction with KIX to these published findings indicates that they could potentially be amenable to medicinal chemistry approaches to increase their specificity for the E2A binding site on KIX, using the NMR structure of KIX bound to E2A solved by our lab.

4.3 Biochemical and biophysical assays to characterize positive hits

Pull-down assays were performed using GST-E2A to pull down full length CBP from RCH ACV nuclear extracts. This was considered a more relevant assay to evaluate the effects of small compounds on protein-protein binding. Relative to the interaction between the synthetic E2A peptide and the isolated recombinant KIX domain, it is likely that the interaction between the full-length proteins depends upon many more contact points. Compound P9 demonstrated successful inhibition of the full-length interaction in pull down experiments at a concentration of 100 μM , the same concentration of the small molecule inhibitor reported by Best *et al* to demonstrate inhibition of the KIX:CREB interaction⁶⁷. With efficacies detectable at the same 100 μM concentration, compound P9 is a respectable small molecule lead compound. It would be valuable to evaluate the inhibitory effects of this compound on purified proteins. Nuclear extracts contain many different proteins that could confound the results. For example, using GST-

E2A to pull down purified, recombinant CBP would be a much cleaner and more specific indication of the effects of compound P9 on E2A-KIX binding.

Proteins in a cell-based assay, as opposed to *in vitro* biochemical assays, are in their native conformations and act in synchrony with other intracellular proteins. Protein-protein interactions occur in various intracellular locations, and it is important to ensure that the inhibitory compound is able to successfully enter the cells through the plasma membrane in order to exert inhibitory effects on the interaction. It is therefore important to test the effects of inhibitory compounds in live cells to ensure that they are exerting equivalent effects as in the *in vitro* assays. Due to the presence of confounding intrinsic fluorescence, solubility problems or generally negative results, compound P9 was the only compound that remained as a viable inhibitor subsequent to performing the biochemical assays. Compound P9 demonstrated an approximate six-fold higher toxicity in E2A-PBX1-expressing cells (the stable 697 cell line and murine myeloid progenitors immortalized with E2A-PBX1) relative to non-E2A-PBX1-expressing REH cells (i.e., EC₅₀ 1 μM versus 6 μM). This marginal difference in cytotoxicity is nonetheless an encouraging result as it is consistent with the ability of P9 to interrupt the full length E2A:CBP interaction. Further studies are required to fully support this finding. Our lab has created two cell lines, 3p and 5p, which are derived from the same progenitor population of flow-sorted pre-B1 cells. Both are infected with a virus conferring expression of the constitutively active tyrosine kinase BCR-ABL; 5p cells also express recombinant E2A-PBX1. The benefits of these cell lines include: 1) since they are derived from the same starting population of pre-B1 cells, at least in theory, the sole difference between them is the expression of E2A-PBX1; and, 2) their B-progenitor immunophenotype makes them representative of the most common form of human ALL. Therefore, future evaluation of P9-associated cytotoxicity in

these cell lines would be more biologically relevant since they are more comparable than those used in this study.

The effects of compound P9 on the E2A:KIX interaction in living cells was also analyzed in a mammalian two-hybrid assay. The results were disappointing in that they did not provide sufficient information on the effects of compound P9 on the interaction. At concentrations up to 10 μ M of compound P9, there was only a slight 10% reduction of luciferase expression as compared to control expression. This decrease in luciferase expression was not statistically significant ($p > 0.37$) and did not provide any information on the *in vivo* protein-protein interaction. At concentrations above 10 μ M, compound P9 began to exert cytotoxic effects on the cells, therefore higher concentrations of compound P9 could not be tested. This could be a limitation in observing effects of compound P9 on the interaction in live cells. Due to cytotoxic effects of P9, and other unknown factors, the results of this assay remain inconclusive and a different cell-based assay would be beneficial.

Fluorescence resonance energy transfer (FRET) is a method that has been used successfully to detect protein-protein interactions in living cells, and could serve as a more direct method of analyzing the effects of compound P9 on the interaction⁷³. Fusing two different fluorophores with overlapping emission and absorption spectra (donor and acceptor) to two proteins of interest allows for the real-time detection of the interaction. N-terminal fusion of one of these chromophore pairs, for example cyan fluorescent protein and yellow fluorescent protein, to KIX and E2A would result in quenching of the donor fluorescence and an increase of fluorescence at the acceptor emission wavelength upon interaction. The effects are ascertained using fluorescence microscopy; the ability of compound P9 to cause a reduction of the acceptor fluorescence would indicate inhibition of the interaction. This visual method is advantageous as it allows for determination of the cellular localization of the GFP-labeled proteins. Furthermore,

the signal is a direct result of the physical approximation of the two proteins in that it does not rely on other interactions or chemical or biological processes.

Compound P9 demonstrated cytotoxicity in E2A-PBX1 positive and negative leukemic cell lines at 1 μM or 6 μM , respectively. However, it only demonstrated inhibition of the E2A:CBP interaction at a much higher concentration of 100 μM . It is unknown what the concentration of CBP is in the RCH ACV nuclear extract used for the pull downs in the current study. Using purified proteins for pull down assays would be more indicative of the efficacy of compound P9 in the *in vitro* inhibition of the interaction. It is also unknown if P9 was able to enter cells to exert its effects, or if exerted cytotoxic effects via an extracellular mechanism of action. A different cell-based assay to directly determine inhibitory effects of P9 and to monitor cellular uptake, such as FRET, would therefore be useful to further characterize P9. These results could be compared to the ability of P9 to inhibit the interaction of purified GST-E2A and CBP proteins in a pull down assay. This would give a more detailed comparison of the *in vitro* and *in vivo* mechanisms of action of compound P9.

4.4 Implications of small molecule inhibitors for ALL treatment

Current research for the treatment of ALL includes the development of superior drugs to target the genetic alterations found in specific subtypes. Tailoring treatments to patients will significantly reduce the side effects and toxicity of chemotherapeutic agents, which are non-specific and harmful to patients. The drugs currently used to treat ALL patients include GCs, vincristine and anthracyclines. These have many side effects due to their nonspecific nature. For example, many chemotherapeutic agents act on rapidly dividing cells to inhibit DNA replication or mitosis. The use of specifically targeted inhibitors has shown great promise in cancer research, forming tight interactions with their specific protein or receptor target. Many inhibitors have demonstrated positive results; improved quality of life, increased overall survival, with some

resulting in complete remission and improving the cure rate of the disease. Although some specific inhibitors demonstrate mild efficacy as a single agent, such as all-trans retinoic acid (ATRA) for the treatment of acute promyelocytic leukemia (APL)⁷⁴, they are given as part of a combination treatment with other chemotherapeutic agents⁷⁵. This would most likely be no different for an efficient inhibitor of E2A-PBX1:CBP in ALL. Although the inhibitory compound acts on the interaction that has been implicated in oncogenesis, there are other genetic events that play a role in the development of a leukemic clone. In order to eradicate the entire progeny of lymphoblasts, an additional chemotherapeutic agent is administered. This combination treatment would be far less toxic to the patients than a multi-chemotherapeutic regimen.

4.5 Potential utility of small molecule inhibitors for understanding E2A-PBX1 biology

Beyond its potential utility in treating patients, an effective inhibitor of the E2A-PBX1:CBP interaction would be a useful tool in studying the biological effects and functional consequences of the E2A-PBX1 chimeric protein and its interaction with CBP. E2A-PBX1 knockdown studies have been performed using siRNA, contributing important information about the biological consequences of E2A-PBX1⁷⁶. Small molecule inhibitors are potentially advantageous relative to the siRNA approach. For example, small molecules are possibly more easily delivered to their target as compared to siRNAs, which are structurally rigid molecules that are relatively difficult to deliver to the targeted cells⁷⁷. siRNA is able to silence the gene of interest with relatively high specificity, however to treat ALL cases it would need to be administered systemically, requiring substantial siRNA to get sufficient knock-down.

A small molecule inhibitor of E2A-PBX1:CBP would be indirectly inhibiting the recruitment of the co-activator complex mediated by CBP, required for the acetylation of lysine

residues on histone tails and consequent transcriptional activity. A HAT assay would determine if the inhibitor resulted in a decrease in acetylation. Theoretically, the inhibitor should be interfering with the ability of the co-activator complex to acetylate histones, and therefore a decrease in acetylation of chromatin in the general area to which CBP is recruited should be observed. Many genes are expected to be differentially regulated downstream of E2A-PBX1 and its recruitment of CBP. For example, the aberrant transcriptional activation of PBX/HOX target genes has been implicated in oncogenesis mediated by E2A-PBX1⁷⁸. Inhibiting the oncogenic E2A-PBX1:CBP interaction with a small molecule would facilitate experimental investigation of the subsequent effects on the regulation of downstream genes. For example, gene expression profiling using microarrays to compare mRNA transcripts in wild type lymphoblasts versus lymphoblasts treated with an inhibitor would provide important information on which genes are differentially regulated and, consequently, should facilitate elucidation of the molecular mechanisms by which this deregulation occurs. Such an inhibitor would theoretically reverse at least some of the E2A-PBX1-driven transcriptional deregulation, as was observed after E2A-PBX1 knockdown using siRNA⁷⁹. Casagrande *et al* observed a substantial decrease in up-regulated genes after E2A-PBX1 siRNA treatment. Similar results could be expected after inhibition of the interaction with small molecules. In their study, Casagrande *et al* also found that E2A-PBX1 knock down in cells resulted in an increase in apoptotic markers, indicative of programmed cellular death⁷⁹. If cells expressing the t(1;19) translocation are dependent on E2A-PBX1 expression for survival, then inhibiting crucial interactions involved in its activity could result in apoptosis. An apoptosis assay could determine whether treatment of E2A-PBX1-dependent cells with a small molecule inhibitor results in cellular apoptosis.

ALL patients expressing the t(1;19) translocation demonstrate many cells which are blocked at the pre-B stage of differentiation. Treatment of E2A-PBX1-expressing cells with an

inhibitor of the E2A-PBX1:CBP interaction should alleviate this block in differentiation if it is caused by differential regulation of genes involved in B cell development. The effects of the small molecules on the differentiation of pre-B cells can be determined by immunophenotyping for B cell markers expressed specifically at various stages of development. The inhibitor would also be expected to affect cellular proliferation rates of E2A-PBX1-expressing cells. Cells expressing E2A-PBX1 with the L²⁰A mutation, which inhibits the E2A-PBX1:CBP interaction, lose the ability to proliferate indefinitely in media. With the same functional consequences as the L²⁰A mutation, the small molecule inhibitor should have the same affect on cellular proliferation. Thus, one might predict that treatment of E2A-PBX1 leukemia cells with an effective small molecule antagonist of E2A:KIX binding should induce maturation of leukemic cells beyond the pre-B-cell stage and/or induce apoptotic death.

4.6 Future directions

In this study, a lead compound, denoted P9, has been identified for inhibition of the E2A-PBX1:CBP interaction. It binds to an E2A-derived peptide with a 20 μ M K_D , an affinity comparable to inhibitors found by other groups from high throughput screening. The next step is to increase the specificity and affinity of the compound for its target sequence in E2A. Structural biologists have solved the structure of the KIX domain, and recently in our lab the structure of a complex consisting of the KIX domain bound to an E2A peptide has also been determined (**Figure 1-5**). With NMR spectra representing both the KIX domain alone and the KIX domain bound to the E2A peptide, we have a powerful tool for investigating the effects of the small molecules on the interaction. We can determine interactions between either isotopically labeled KIX or E2A and the compounds of interest by examining if any of the amino acid residues shift upon the addition of the small molecules. Having mapped the structure of KIX bound to E2A, it is possible to do rational drug design using the leads obtained from the high throughput screens,

such as compound P9 which interacts with E2A, or compounds L1 and C2 which bind to KIX. It would be beneficial to map these small molecules onto the E2A or KIX domains to determine where they dock on the peptides. The specificity of these interactions could then be increased using medicinal chemistry approaches. Altering the side groups of the small molecule to obtain a tighter interaction with the hydrophobic cleft of the KIX domain or the helical portion of the E2A peptide would increase both the affinity of the small molecule for the domains, and possibly the specificity of the small molecule for the KIX:E2A interaction. The specificity of P9 binding to E2A will probably require improvement. Furthermore, the current 20 μM K_D suggests an insufficient affinity for clinical use. Compounds used to treat human cancer cases interact with their target with K_D values in the low nanomolar range. Examples are all-trans retinoic acid, which interacts with its target PML-RAR α with a K_D of 0.1 nM⁸⁰ and lapatinib, which interacts with the epidermal growth factor receptor (EGFR) with a K_D of 3 nM⁸¹. As the affinity of compound P9 for the E2A peptide is increased by medicinal chemistry approaches, the improvements can be monitored using FA based on the fluorescent signal from the FITC-E2A peptide. Each time the compound is altered, binding curves can be obtained and the K_D values calculated to determine the effect on affinity.

Once a suitable K_D has been obtained, the next step is to test the effects of the improved compound P9 on the development of disease in an animal model. Our lab has developed an *in vivo* mouse model to study the effects of E2A-PBX1 expression. Murine bone marrow expressing E2A-PBX1 are injected into irradiated mice. These mice develop a myeloproliferative disease resembling acute myelogenous leukemia and die within 40 days post-transplantation³¹. The effects of compound P9 on the development or treatment of disease would be analyzed. Information on the safety and efficacy of compounds also are necessary for the use of any compound as a treatment. Pharmacokinetic information must then be obtained, such as

information on absorption, distribution, metabolism, excretion and toxicology (ADMET). Due to the vast differences between the physiology of mice and humans, many lead compounds fail in phase I clinical trials due to safety concerns, supporting the requirement for intensive studies in better, more informative animal models before the clinical trial stage. Humanised ADMET mice have recently been developed (CXR Biosciences, TaconicArtemis, ITI Lifesciences) which incorporate important human genes that are involved in the ADMET process⁸². This reduces interspecies variability and provides important information on the pharmacokinetics of the compound, before it is subjected to clinical trials.

4.7 Significance

In this study, the development of an FA-based binding assay for the E2A-PBX1:CBP interaction, screening with small molecule libraries, and a series of assays to characterize and evaluate positive hits has resulted in the successful discovery and characterization of a lead inhibitor. Not only does compound P9 demonstrate inhibition of both the shorter E2A and KIX peptides and the longer E2A fragment with the full length CBP protein, but it also demonstrates cytotoxic effects in cells dependent on E2A-PBX1 for survival, and interacts with the E2A domain with a 20 μM K_D . The novel protocol developed (**Figure 3-1**) is a series of assays which can be exploited for the discovery of a small molecule inhibitor of any interaction that is dependent on a small focal point. It provides detailed and thorough methods, from the development of a binding assay amenable to high throughput screening, through the various stages of characterizing positive hits and removing compounds which show negative results. The discovery of lead compounds for the treatment of cancer is an important aspect in cancer research. These inhibitory compounds can be exploited for use in basic cancer research, to gain knowledge of the downstream effects of the protein-protein interaction. Knowledge of the mechanisms of cancer development and the downstream effects of oncogenes and the various

interactions they form will aid in the battle against this disease. Inhibitory compounds can also potentially be used in the therapeutic treatment of disease in humans, which would present novel specific treatments for subsets of cancer cases.

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