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Understanding and targeting the C-terminal Binding Protein (CtBP) substrate-binding domain for cancer therapeutic development

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

By

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Abstract:

Cancer describes a condition involving the dysregulated proliferation and growth of cells throughout the body. Among other causes, the etiology of cancer consists of various insults and abnormal activation of factors that can provide a growth or survival advantage to the cancer population. One family of genes, C-terminal binding proteins (CtBP) 1 and 2 are transcriptional co-regulators upregulated in several cancers, including a majority of studied breast, colorectal, and ovarian tumor samples. CtBPs drive many cellular oncogenic properties, including migration, invasion, proliferation, and survival, in part through repression of tumor suppressor genes. CtBPs encode an intrinsic dehydrogenase activity regulated by both intracellular NADH concentration and the putative substrate 4-methylthio-2-oxobutyric acid (MTOB), which regulates the recruitment of transcriptional regulatory complexes based on dimerization induced by bound NADH. High (millimolar) levels of MTOB appear to inhibit CtBP dehydrogenase function and induce cytotoxicity among cancer cells in a CtBP-dependent manner. While encouraging, a good therapeutic would involve drug concentrations at least two orders of magnitude lower. To this end, a new endeavor was undertaken to discover and design a first generation set of therapeutics that are CtBP-specific and cytotoxic. The best of these drugs, known as 3-Cl and 4-Cl HIPP exhibit high nanomolar enzymatic inhibition and high micromolar cytotoxicity. As a bi-product of our studies we have determined that CtBP enzymatic function is subject to allosteric interactions.

The function of the substrate-binding domain has yet to be examined in context of CtBP's oncogenic activity. To this end, we have created several point mutations in the substrate-binding

pocket of CtBP and have determined key residues for CtBP's enzymatic activity using an *in vitro* dehydrogenase assay. We have found that a conserved tryptophan in the catalytic domain, and specifically its aromatic moiety, is imperative for enzyme activity. This tryptophan is unique to CtBP family proteins and distinguishes the CtBP substrate-binding domain from that of all other families of dehydrogenases. Additionally, we found arginine and histidine residues lining the substrate pocket that are necessary for CtBP dehydrogenase function. Knowledge of these residues allows the directed synthesis of drugs with increased potency and higher CtBP specificity. Early work has been performed to understand the importance of these mutations in cancer phenotypes. Taken together, this work interrogates the utility of CtBP, and specifically the CtBP substrate-binding domain, as a target for cancer therapeutics. We also provide preliminary insights into the function of this domain enzymatically and in cellular models of cancer, showing that inhibitors targeted to the CtBP-binding domain have potential for future clinical utility.

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List of Abbreviations:

A.A.: Amino acid	Kcat: Turnover number
APC: Adenomatous polyposis coli	kDa: kilodaltons
ARF: Alternate reading frame	Ki: Inhibition constant
BRCA1: Breast Cancer 1	Km: Michaelis constant
BRK: Baby rat kidney	MEF: Mouse embryonic fibroblast
ChIP: Chromatin immunoprecipitation	MTHB: 4-methylthio-2-hydroxybutyrate
CtBP: C-terminal Binding Protein	MTOB: 2-Methylthio-3-butyric acid
D2-HDH: D-isomer-specific 2-hydroxyacid dehydrogenases	MTS: (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)
EC50: Effective concentration (at 50% activity)	MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
EMT: Epithelial-Mesenchymal transition	NADH: Nicotinamide adenine dinucleotide (reduced)
FAP: Familial adenomatous polyposis	NLS: Nuclear localization signal
FBDD: Fragment based drug design	Ppy: Phenylpyruvate
FRET: Fluorescence Resonance Energy Transfer	pRb: Retinoblastoma protein
GST: Glutathione S-transferase	PTEN: Phosphatase and tensin homolog
HDAC: Histone deacetylase	PXDLS: Proline-X-Aspartate-Leucine-Serine
HIPP: 2-hydroxy-3-imino-phenylpropionic acid	qPCR: Quantitative polymerase chain reaction
HMT: Histone methyl-transferase	SBHA: Suberohydroxamic Acid
IC50: Inhibitory concentration (at 50% activity)	Vo: Initial velocity
ITC: Isothermal calorimetry	Vmax: Maximum velocity
IP: Intraperitoneal	

Chapter 1: Introduction:

Discovery of the C-terminal Binding Protein family:

C-terminal binding proteins were first discovered in 1993 as part of an investigation to differentiate the portions of the E1A adenoviral proteins that were necessary for transformation.^{1,2} E1A proteins are a well-studied type of 2/5 adenovirus capable of immortalizing primary rodent cells and manipulating cellular growth, survival, and differentiation in order to enhance viral gene replication and gene expression in mammalian cells.³ Interestingly, early studies illustrated a discrepancy of function between different domains of the protein. E1A proteins consist of two exons: the first exon cooperates with activated *ras* in baby rat kidney (BRK) co-transformation assays and interacts with other important factors, like the tumor suppressor pRb, to enhance oncogenic potential. Conversely, the second exon can antagonize transformation, tumorigenesis and metastasis in rat models. Deletions of the C-terminal 67 amino acids of E1A showed a ‘super-transformation’ phenotype in *ras* cooperative transformation assays in immunocompromised mice, while simultaneously impeding BRK immortalization.¹ To find factors that may contribute to this phenotype, a Glutathione-S-transferase (GST)-C-terminal E1A fusion protein was created, transfected into HeLa cells and eluted off glutathione-agarose beads to find a dimer of protein bands that ran at 48kDa on an agarose gel. This protein was aptly called C-terminal binding protein, or CtBP¹. While E1A was predominantly considered an oncoprotein at the time, in this instance the converse was found to be true: E1A acts as a tumor suppressor to repress CtBP oncogenic activity, specifically by preventing CtBP from inducing an Epithelial to Mesenchymal phenotype transition (EMT).⁴⁻⁶ Following this initial discovery, a wealth of information has been discovered about CtBPs, their conservation, biological roles, and roles in cancer.

Biological roles of CtBPs and their evolutionarily conserved homologs:

CtBP family proteins are a highly conserved family of transcriptional co-regulators that are crucial for normal development and homeostasis. CtBPs and their orthologues have been found to be conserved among several different eukaryotic species (**Fig. 1_1**).^{7,8} In plants, the orthologous gene *ANGUSTIFOLIA* (AN) is important for regulating stress responses, the timing of flowering, seed production, and microtubule polarity that, in turn, regulates leaf width.^{9,10} In *C. elegans* *ctbp-1* expression has been found to play a key role in permitting acute functional tolerance of ethanol, lipid regulation through repression of a lipase, *lips-7*, and increasing lifespan.^{11,12} Additionally, it maintains an important role in development of proper axonal morphology of SMD dorsal neurons and exploration behavior.¹³ Research in drosophila further validated the need of CtBP (or dCtBP) in development. As an embryo develops, it relies on a menagerie of different co-repressors, including dCtBP, to properly determine segmentation. The drosophila model gives insight into the many context-dependent nuances of gene regulation generally, and of the role of CtBP specifically. dCtBP co-repression helps determine dorsal-ventral patterning and plays a role in segmentation from the anterior to posterior. This depends the genes regulated, and the regulation site. For example, dCtBP can work with the repressor Kruppel to prevent transcription of a gene known as *even-skipped* at the second stripe of segmentation but not at the fifth. Tangential to these observations, embryos lacking maternal dCtBP show severe patterning defects.¹⁴ Additionally, in some instances in drosophila, the oligomerization state of dCtBP can determine if it is a repressor or activator: in one study, monomers of CtBP activate transcription, while dimers follow canonical repression.^{15,16} Past the point of early development, dCtBP has been shown to help activate genes associated with

circadian rhythm maintenance and participate in eye development and maintenance, among other regulatory functions.^{17,18}

Distinct from invertebrate models which only have one CtBP-family gene, vertebrates have two members, named CtBP1 and CtBP2. Mouse models involving CtBP knockouts show that CtBPs are similarly imperative in mammals as they are in drosophila: CtBP1 null mice are small but viable. CtBP2-null mice, however, do not live past embryonic day E10.5. The genes have a somewhat compensatory effect, such that loss of a single allele of CtBP2 within CtBP1 null mice causes death in utero and shows multiple developmental defects. Eliminating a single CtBP1 allele in CtBP2 null embryos inhibits neural tube closure. Looking past the initial stages of development, it was shown that CtBP is prevalent in the nervous system, eyes, ears, pharyngeal arches, muscles, and limbs of mice fetuses.¹⁹ A study in quail embryos served to validate the role of CtBPs in vertebrate development in another model: specifically, CtBPs are imperative to limb bud development and neural development, with CtBP1 and CtBP2 having both unique and redundant roles.²⁰ Participation in various other roles have been ascribed to CtBPs, including brain and synapse localization, mitochondrial maintenance (through Bax repression), and fat differentiation by repressing white fat genes.^{2,21-24} Our concern with CtBPs, however, has to do with their increasingly indicated role in cancer and oncogenesis, as discussed further below.

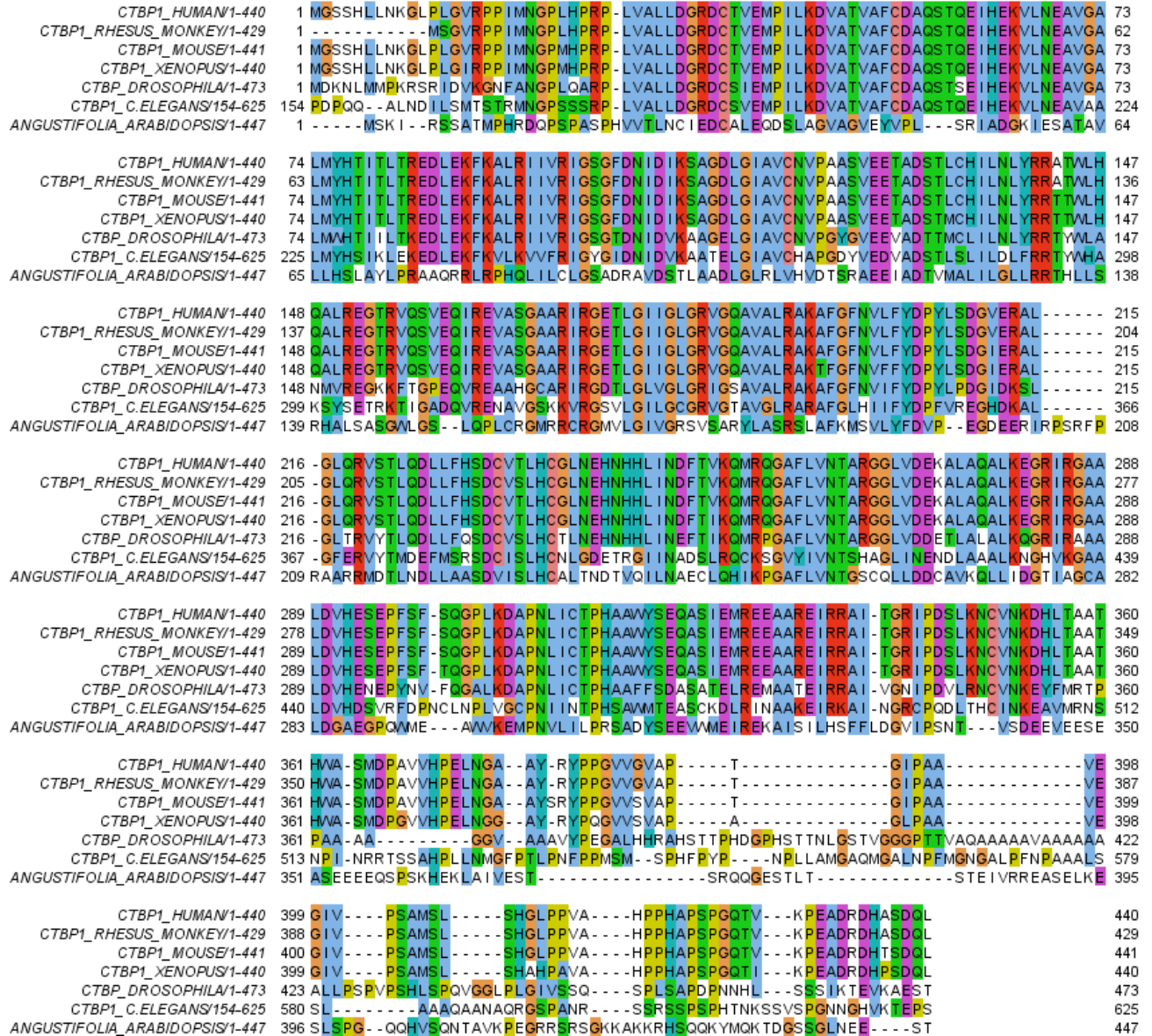


Figure 1_1. CtBP is an evolutionarily conserved family of proteins. Using the Cluster Omega tool and Uniprot, sequences were aligned and displayed via Jalview software. Displayed are the protein isoforms for CtBP1 in Homo sapiens (Human), Macaca mulatta (Rhesus monkey), Mus musculus (Mouse), and Xenopus laevis (African clawed frog), along with dCtBP in Drosophila melanogaster (Fruit fly), CtBP-1 in Caenorhabditis elegans (Roundworm), and the homolog ANGUSTIFOLIA in Arabidopsis thaliana (Mouse-ear cress). Sequences are aligned against the entirety of H. sapiens CtBP1.

Function and structure of CtBP1 and CtBP2:

In humans, CtBP1 is located on chromosome 4p16.3 and CtBP2 is found on chromosome 10q26.13; these two genes share 83% similarity.³ Each gene has variants that function outside their primarily studied roles as transcriptional co-regulators. CtBP1 has a variant known as CtBP1-S, referring to the shorter isoform, or CtBP/BARS, which is localized in the cytoplasm and participates in membrane fission from the Golgi complex.²⁵ CtBP2 has three isoforms: CtBP2-S, CtBP2-L, and RIBEYE. CtBP2-S and CtBP2-L are believed to function exclusively as transcriptional regulators. RIBEYE is expressed from an alternative promoter and has two basic domains, the first of which is a unique N-terminal domain, and the second, which is identical with CtBP2. RIBEYE is active only at neurons that contain ribbon synapses and is believed to function, at least in part, as a way to properly attach two different compartments of the synapse.²³ Briefly, ribbon synapses are a special type of synapse at the junction of neurons that are capable of exchanging vesicle pools for a prolonged period of time and at a wide range of gradients. They are often seen where stimuli can similarly be prolonged and variable, such as vision in the eye, and hair follicles of the inner ear.²⁶

CtBP family proteins are unique in comparison to many other transcriptional co-regulators due to the fact that they have intrinsic enzymatic activity. CtBPs most closely resemble D-isomer-specific 2-hydroxyacid dehydrogenases (D2-HDH). Dehydrogenases are capable of performing oxidative-reductive (redox) reactions in which NADH is converted to NAD⁺, or vice versa, in the presence of a co-substrate. As such, CtBPs contain an NADH-binding domain made of a motif consisting of alternating β -strands and α -helices, known as a Rossmann fold, and a substrate-binding domain (**Fig. 1_2B**).²⁷ Additionally, CtBPs have

consistently been shown to homo- and heterodimerize. Another unique feature among CtBPs is their ability to interact with PXDLS (Pro-X-Asp-Leu-Ser) motifs found in several transcription factors and transcription regulators.^{4,28,29} CtBPs have been shown to interact with EGR1/SP1, ETS, CREB, KLF, ZEB, STAT and other families of transcription factors.^{30,31} Through the PXDLS-binding motif various effectors, including CoRest, HDACs, HMTs, etc. can also interact with CtBPs. Some of these factors in turn recruit other effectors. For example, LSD1 (Lysine-specific demethylase 1A) often binds CtBPs, but only through direct binding with CoRest. The effect results in demethylation of gene promoters and transcriptional activation. CtBPs have been found to contain an RRT motif binding domain that allows further binding, but it is redundant with the PXDLS-binding domain; all proteins with a PXDLS domain have been found to have an RRT domain as well.³² It is possible that once the PXDLS occupancy is filled, the RRT cleft may serve to allow a secondary binding site for other components, but the hypothesis requires further validation.²⁸ Finally, there are proteins, such as HDAC2 and HIPK1, that can bind CtBP directly and independently of PXDLS motifs.^{33,34}

With their functions combined, these domains work together to enable CtBPs' transcriptional regulatory capacity (**Fig. 1_2A**). The current hypothesis goes as follows: in order to repress gene promoters, CtBP must localize to the nucleus. In the case of CtBP2, it can translocate to the nucleus through an N-terminal nuclear localization signal (NLS) between a.a. 4-14.^{27,35,36} This localization region is acetylated by p300, which is necessary for nuclear entry.³⁷ CtBP1, however, must rely on other mechanisms. It can bind a DNA-binding transcription factor or other protein containing a PXDLS site to "piggyback" to the nucleus. Alternatively, CtBP1 and CtBP2 can heterodimerize and use the CtBP2 NLS to enter the nucleus.³⁶ Uniquely,

CtBP1, but not CtBP2 contains a C-terminal PDZ-binding site that is believed to be responsible for shuttling CtBP1 to the cytoplasm.³⁸

Once in the nucleus, CtBPs function primarily as dimers or oligomers. While there are prescribed monomeric functions for CtBPs in drosophila,^{2,16,32,33} dimerization is classically required for transcriptional repression.^{16,33,39,40} Dimerization causes a favorable conformational change in the PXDLS-binding domain.^{27,41} PXDLS binding recruits transcription complexes to participate in targeted gene repression. The complex commonly consists of promoter specific transcription factors and effectors, like histone methyl-transferases (HMTs), histone deacetylases (HDACs), and other chromatin remodeling proteins.^{28,29,42,43} A brief graphical representation, illustrating the hypothesis of how this interaction works, can be seen in **Figure 1_2A**. A catalytic triad, consisting of His315(321), Glu295(301) and Arg266(272) in CtBP1 (or CtBP2), allows NADH and a substrate to be brought into close proximity³⁹ (**Fig. 1_2B**). In the presence of substrate, the intrinsic dehydrogenase activity of CtBP converts NADH to NAD⁺. It is hypothesized that CtBP is not enzymatically active *in vivo*,⁴¹ but rather the redox reaction allows disassembly of the transcriptional complex and recycling of CtBP monomers to affect further transcriptional mechanisms.

While this is believed to be the primary mechanism whereby CtBP represses, there have been reported nuances and exceptions to the hypothesis. Dr. Lundblad and colleagues present a report that suggests that CtBP2 may form tetramers, although the relevance to repression is unknown.⁴⁴ Work is still ongoing in the Grossman laboratory and elsewhere to find evidence that this may function in cell culture or *in vivo* systems. Irrespective of this forthcoming information, the basic premise for CtBP transcriptional regulation has been tested and verified.

CtBP as a redox sensor and the putative substrate MTOB

It has been discovered that CtBP's intrinsic dehydrogenase domain functions as a redox sensor, with an ~100 fold preferential binding affinity for NADH over NAD⁺.^{45,46} Thus increased levels of NADH production are correlated with an increase in CtBP-mediated activities.⁴⁶ An increase in the NADH:NAD⁺ ratio is especially observed in cancer systems and may be attributed to at least two promulgating factors: hypoxia and changes in metabolism, such as via a phenomenon known as the Warburg Effect. The Warburg effect describes the tendency that cancer cells have to preferentially undergo a process similar to anaerobic glycolysis, a method of energy production that normally occurs in low oxygen conditions, leading to lactate and NADH production, regardless of oxygen availability.⁴⁷ Similarly, hypoxic conditions compound glycolysis, resulting in a further increase in available NADH.⁴⁸ Hypoxia, in turn, often predisposes neoplasms to become metastatic.⁴⁹ The end result of these processes explains the observation that CtBPs are often overexpressed in malignancy.⁵⁰

Investigators were unaware of the substrate involved in CtBP enzymatic activity until 2007, when Dr. Younes Achouri and colleagues performed a screen of potential cofactors. They found the putative substrate to be 2-keto-4-methylthiobutyrate (also referred to as 4-methylthio-2-oxobutyric acid or MTOB).⁵¹ MTOB principally functions as the penultimate intermediate in the methionine salvage pathway, a pathway involved in the natural recycling of methionine side chains throughout the body. In relationship to CtBP, it is a specific and 80-fold better substrate for CtBP's dehydrogenase activity than other structurally similar compounds. Interestingly, MTOB demonstrates biphasic kinetics in the presence of CtBP; low levels of MTOB serve as a substrate to promote CtBP function, while high levels are inhibitory.⁵¹ This inhibitory function provides the basis for therapeutic development, discussed in the rationale of Chapter 3.

CtBP function in a cancer context:

An understanding of the normal prescribed functions of CtBPs in cell biology and their mechanism of action are fundamental for studying this family of proteins within the context of human disease. Specifically, CtBPs have been indicated as a player in oncogenic transformation and cancer survival. Work headed by Dr. Evan Sumner in the Grossman laboratory (a review extends beyond the scope of this introduction), demonstrates that CtBP family proteins function as oncogenes. At the level of transcriptional regulation, CtBPs promote and maintain several cancer functions. In order to fully understand this regulation, it is important to review a few of the hallmarks of cancer. Cancer consists of dysregulated cell proliferation within the body and several hallmarks that characterized cancer have been described by Drs. Hanahan and Weinberg.⁵² Their framework states that most cancers must sustain proliferative signaling to continually divide, evade growth suppressors that would ameliorate proliferation, enable replicative immortality, resist cell death via apoptosis or other death pathways, activate invasion and metastasis, and induce angiogenesis. Further, cancer cells are capable of deregulating normal cellular energetics in order to favor pathways, such as described with the Warburg effect, and are able to avoid destruction via the immune system.⁵² Studies have implicated CtBPs in properties that tie into these hallmarks such as cell migration, invasion, cell survival, metastasis, metabolic maintenance, and stem cell pathways.

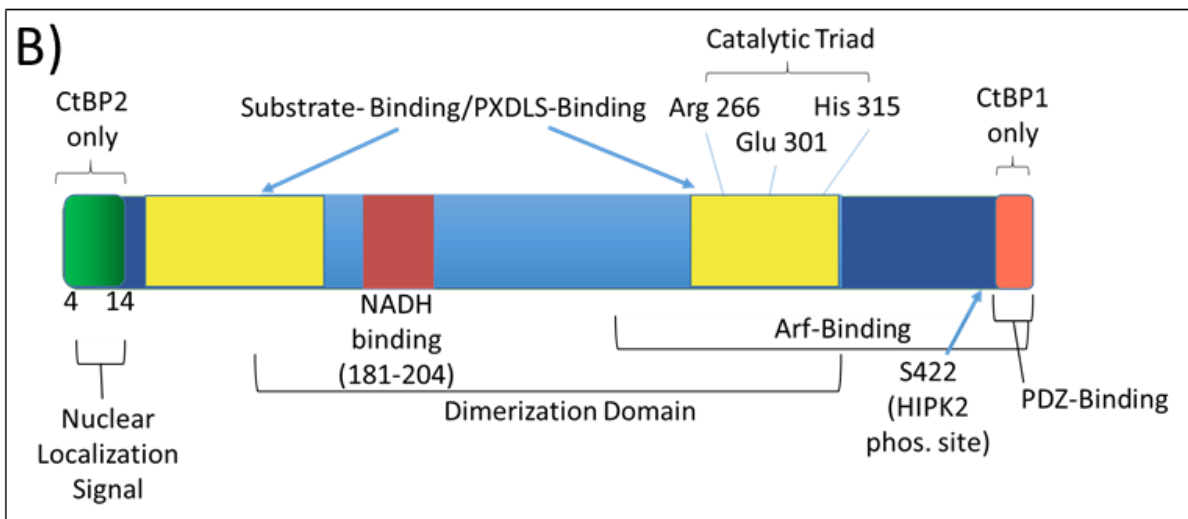
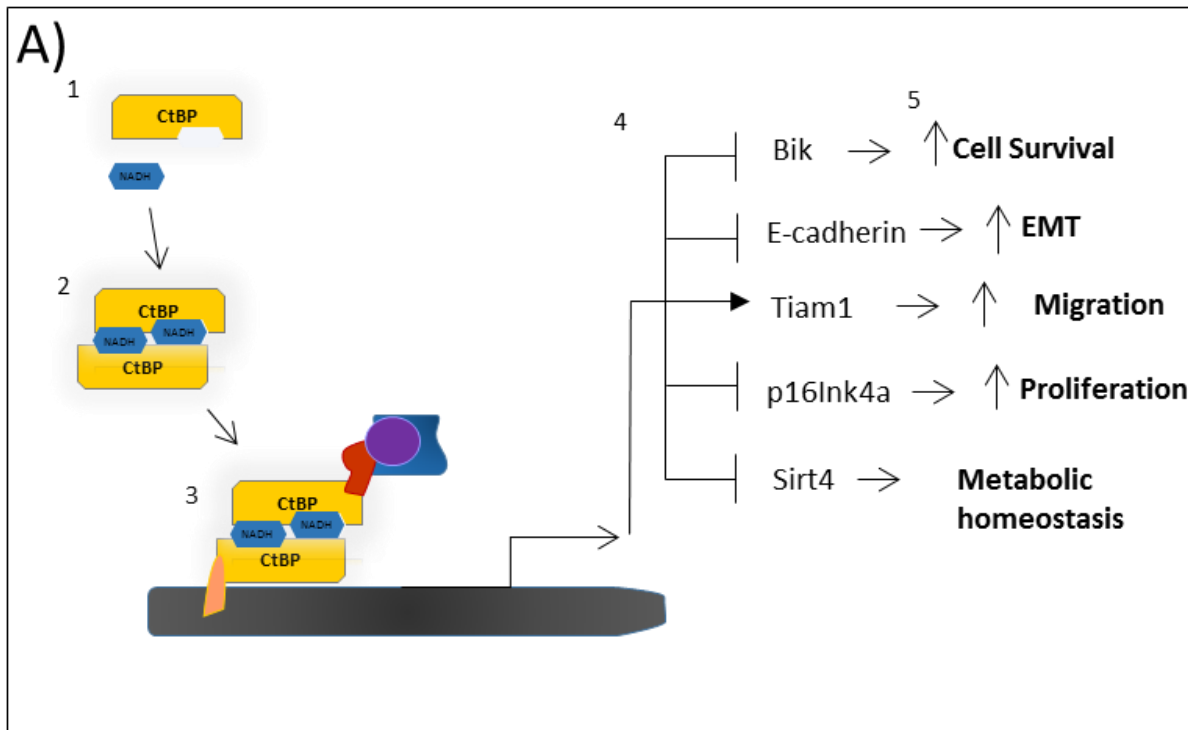


Figure 1_2. CtBPs' structure allows for the recruitment of transcriptional complexes that promote cancer phenotypes. **A)** The hypothesized mechanism of CtBP oncogenic potential. In the presence of NADH (1), CtBP homodimerizes (2), allowing a conformational change that recruits PXDLS partner binding of transcription factors and effectors (3), which leads to repression of multiple targets (including tumor suppressors) (4) that lead to increased cell survival, migration, invasion, proliferation, metabolic homeostasis, etc. (5). Examples of transcriptional targets are shown for each phenotype. The role of substrate is unknown. **B)** A basic representation of key features of CtBPs in the long isoform. Features unique to either CtBP1 or 2 are emphasized.

Substantial work has been performed to distinguish the genes and pathways that CtBPs regulate within the context of cancer. For example, CtBPs promote epithelial to mesenchymal transition (EMT) by transcriptionally repressing epithelial genes, like E-cadherin and keratin-8, and activating the pro-invasion gene Tiam1.^{53,54} The epithelium is the tissue structure that forms the outer most layer of the body and also lines organs and vessels. In contrast, mesenchymal embryonic cells develop into connective tissue or lymphatic and circulatory cells. This epithelial to mesenchymal transition permits cancer to spread and proliferate by becoming more migratory. The process of EMT involves, among other changes, the loss of E-cadherin, which is integral for maintaining cell adhesion through adherens junctions. Keratin-8 encodes an intermediate filament that is specific to epithelial cells, and its loss promotes de-differentiation from the epithelial phenotype. Beyond promoting migration and invasion, CtBP also regulates the tumor environment by maintaining pH and metabolic homeostasis through negative regulation of SIRT4.⁵⁵ SIRT4 prevents a process called glutaminolysis that functions to normalize intracellular acidification under conditions of glycolysis in cancer. Thus downregulation of CtBP lowers the pH of cancer cells by allowing SIRT4 to be expressed.

Importantly, CtBPs also encourage pro-growth and pro-survival oncogenic characteristics. CtBP null mouse embryos demonstrated an upregulation of proapoptotic genes, such as PERP, Noxa, Bax, and Bik.^{53,56} In particular, Bik is an especially integral target of CtBP repression. Work has shown that transient knockdown of Bik by siRNA rescues CtBP-mediated cell death.⁵⁷ When Bik is expressed, it localizes to the endoplasmic reticulum (ER) to release calcium ions (Ca^{2+}). This release signals a mitochondrial fusion gene, DRP1, to localize to the mitochondria and open mitochondrial cristae structure. Simultaneously, Bik promotes mitochondrial localization of two pro-apoptotic genes, Bak and Bax. DRP1, Bak, and Bax all

work to release a signaling molecule, cytochrome c, from the mitochondria, which then in turn activates caspase-9, a protease that induces apoptotic cell death.⁵⁶ CtBP represses other known tumor suppressors including p16Ink4a, p21, PTEN, and p15Ink4b.^{3,29,53} For example, p16Ink4a functions as a cell cycle checkpoint that can stall G1 to S phase progression (the point in which DNA is replicated) in cell division. CtBP recruits factors to repress p16Ink4a expression, which helps cells to bypass stalled replication or senescence.^{58,59} PTEN (Phosphatase and tensin homolog) is a potent tumor suppressor that prevents aberrant cell cycle progression, survival and homeostasis by repressing a cascade of biological processes that are catalyzed by the PI3K/AKT pathways. Additionally, CtBPs affect a variety of other tumor suppressors and gene targets, causing an increase in pro-oncogenic functions in cancer cells as the global end result.

Our understanding of the CtBP expression profile in cancer was thrust forward by ChIP-seq (chromatin immunoprecipitation sequencing) experiments that validated these findings and provided other novel insights. In MCF-7 breast cancer cells, CtBPs, outside normal regulatory roles, play significant roles in assuring EMT, cell cycle progression, proliferation, and survival³⁰ (**Fig. 1_2A**). Furthermore, these ChIP-seq experiments validated roles of CtBP in restricting cellular repair (i.e. repression of BRCA1) and promoting stem cell-like features, etc.³⁰ The increased repression of the aforementioned targets, especially under hypoxic and increasingly oncogenic conditions lends weight to CtBP as a putative oncogene.^{29,60}

In addition to suppressing tumor suppressors, CtBP is also subjected to tumor suppressor regulation. In particular, ARF and HIPK2 are capable of causing p53 independent degradation to abrogate pro-tumorigenic functions. In response to UV radiation, HIPK2 phosphorylates CtBP and targets it to the proteasome by increasing ubiquitinated CtBP. This HIPK2-mediated proteasomal localization causes CtBP degradation and promotes apoptosis.^{61,62} ARF binds the

C-terminal portion of the CtBP protein (within a.a. 224-445 in CtBP2, **Fig. 1_2B**) to relieve CtBP's inhibition of Bik, which induces apoptosis through a p53-independent pathway involving proteasomal degradation.^{56,57,63-65} This is critical, since p53 deregulation is involved in most tumor formation.⁶⁶ Interestingly, ARF expression does not regulate CtBP by increasing CtBP ubiquitination. The exact mechanism wherein ARF induces CtBP degradation has yet to be elucidated, but one hypothesis suggests that ARF can itself be targeted to the proteasome by ubiquitination, and that its own proteasomal localization causes the degradation of any partners to which it may be bound.⁶⁷ It has been shown that ARF can sequester itself, along with CtBP, to the nucleolus, suggesting that its ability to shuttle CtBP may in part dictate CtBP regulation.⁶³ Alternatively, ARF has been shown to regulate sumoylation of binding partners, which similarly can target its binding partners for proteasomal degradation.⁶⁸ ARF-dependent sumoylation of CtBP has not been investigated. Beyond apoptosis, ARF can also block CtBP mediated PTEN repression. Alleviation of PTEN function allows for repression of the PI3K/Akt pathway; the global effect results in inhibition of CtBP-dependent, hypoxia-induced, cancer cell migration.⁶⁴ A third mechanism of CtBP repression can be seen with the tumor suppressor APC. It was shown that patients with Familial Adenomatous Polyposis (FAP), a condition caused by germline mutations in APC, had higher levels of CtBP1 in adenomas compared to normal tissue.^{29,69} It was further found that APC rescue in colorectal cell lines caused diminished CtBP through proteasome-dependent mechanisms.

The evidence for CtBP-specific oncogenic potential has been studied largely in human cancer-derived cell lines and animal models. Ultimate proof of its potential clinical utility resides in effects seen in cancer in the patient population. The Grossman lab performed one of the first studies looking at differential expression of CtBPs in a tumor versus control tissue cohort. In a

study of 69 human colorectal adenocarcinoma samples, our lab found 64% to show upregulated levels of CtBP when compared to normal adjacent tissue sections from the same patients⁷⁰. Even more recently, a study has shown that CtBP1 is more highly expressed in certain forms of breast cancer, where it is expressed in 92% of invasive ductal breast tissue samples but expressed in only 4% of normal adjacent tissue.⁷¹ A connection between increased CtBP1 and decreased E-cadherin and BRCA1 protein was also verified within the tumor samples.

CtBP2 is also a proposed oncogene in ovarian cancer. One study showed that 83% of ovarian epithelial tumors demonstrated increased CtBP2 expression when compared to normal and benign ovarian tissues.⁷² CtBP2 expression scored higher in invasive tumors than that of borderline tumors. Survival of patients who had CtBP2-positive tumors was lower than that of patients with negative expressing ovarian tumors, based on Kaplan-Meier curves. In prostate cancer, CtBP2 expression similarly correlates with increased cancer severity.⁷³ Comparing the extremes of poorly differentiated prostate cancer with normal prostate tissue, elevated CtBP2 expression was observed in 80% and 6% of samples, respectively. Kaplan-Meier curves likewise showed a statistically significant lower survival rate in patients with high CtBP2-expressing tumors. Research continues to find pathways in which CtBPs play a pivotal role in the development of many cancers and to distinguish which cancer types are most regulated by this protein family.

Using the Cancer Genome Atlas (TCGA), an NIH sponsored cancer genomic data set, it is possible to interrogate the genomic and transcriptional events that lead to overexpression in several of these cancer types. Generally, CtBP1 or CtBP2 genomic alterations are found in a small percentage of tumors, with the highest frequencies including ovarian and uterine cancers (**Fig. 1_3**).^{74,75} While this can explain some of the overexpression seen in tumor samples,

genomic amplification seen in a minority of cancers does not fully account for the overexpression seen in a majority of tumor samples in breast, ovarian, and other cancers. Nor does the transcriptional expression profile in these tumors: CtBP1 seems to be ubiquitously expressed at fairly consistent levels (**Fig. 1_4A**), while CtBP2 expression varies much more (**Fig. 1_4B**). Anecdotally, CtBP2 transcription seems to correlate somewhat with immunohistochemistry and protein expression experiments; for example, in the TCGA gene expression data (**Fig. 1_4B**), colorectal, prostate, ovarian, and breast tumors are among the highest six CtBP2 expressing cancer types. This falls in line with the aforementioned data. It is possible though, that most of the increased expression of CtBPs in cancer is due to posttranscriptional protein maintenance and stability that provides an oncogenic advantage.

To summarize, while CtBPs are crucial for normal development, they also participate in cancer proliferation and survival through several different pathways. The relevance of CtBP overexpression is increasingly being reported in the patient population. The subsequent experiments in this dissertation endeavor to discover new and better therapeutics that can successfully target and kill cancer cells in a CtBP-specific manner and to further understand the mechanism of CtBP transcriptional and biological function, specifically with regard to its substrate-binding activity.

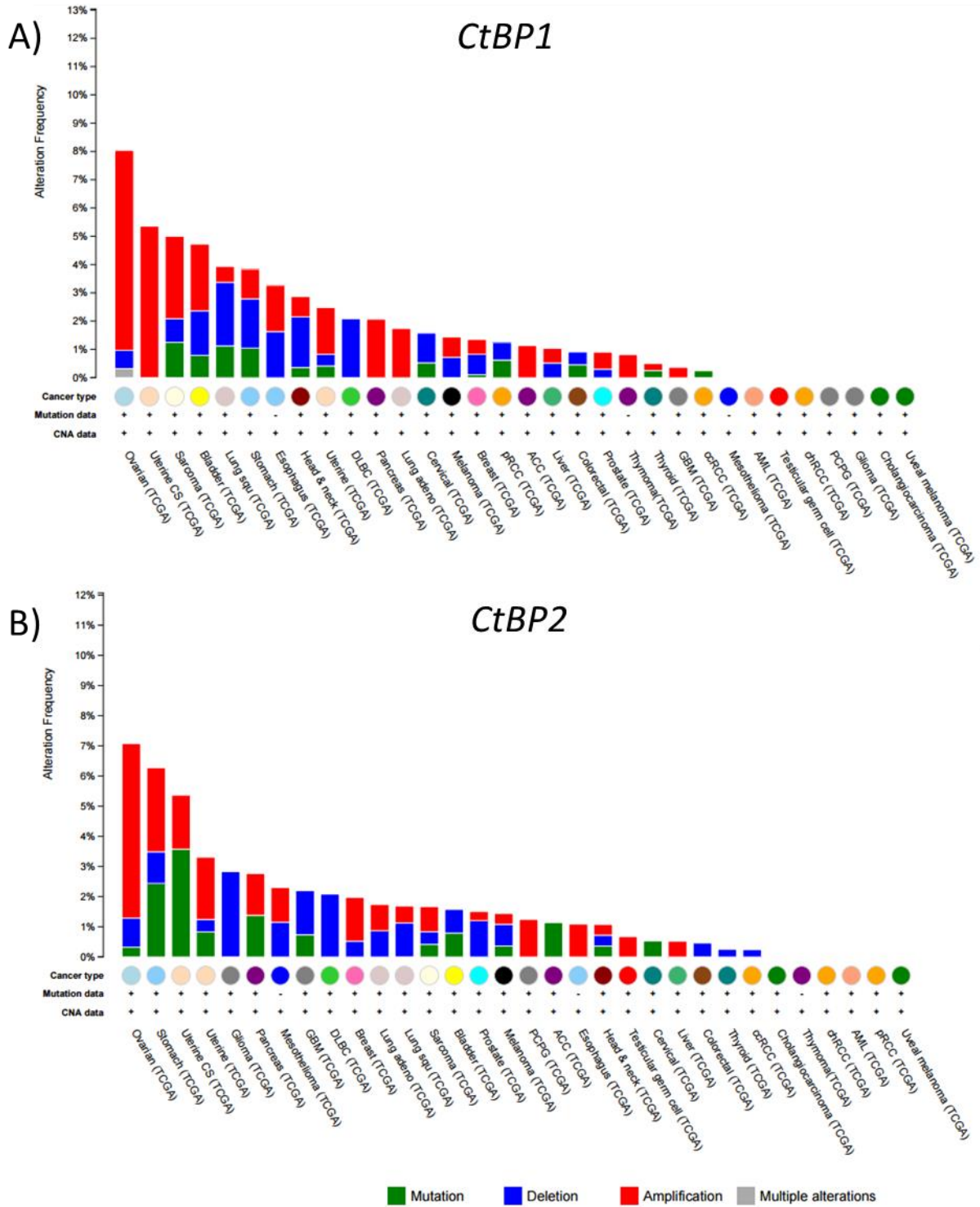


Figure 1_3. CtBP1 and 2 differ in genomic alterations among different cancer tissues. Data shows genomic differences for A) CtBP1 and B) CtBP2 across a panel of provisional tumor TCGA tissue samples, arranged in order of alteration frequency. These results are based upon data generated by the TCGA Research Network: <http://cancergenome.nih.gov/>.

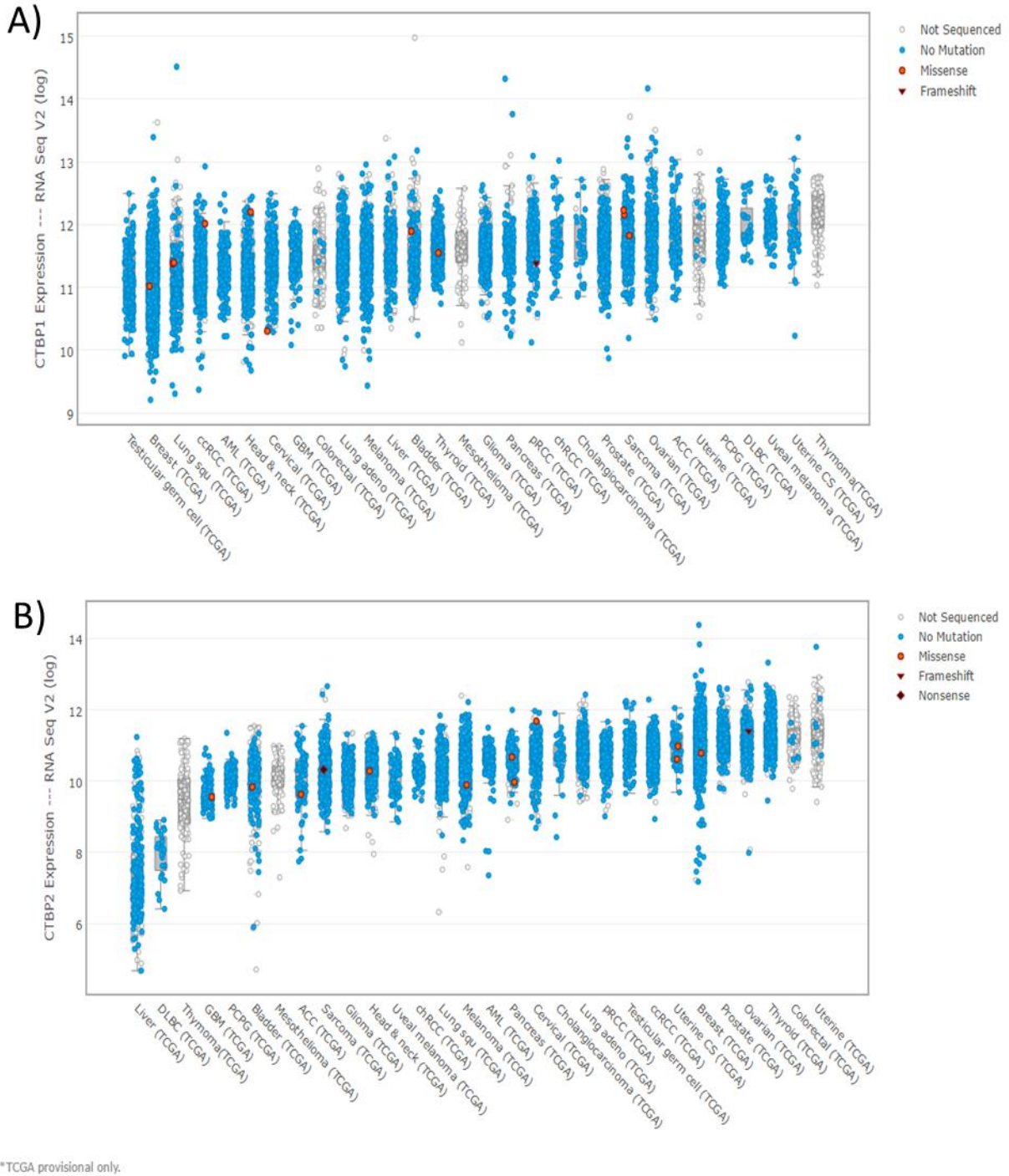


Figure 1_4: CtBP1 and 2 show differing trends in gene expression across tumor types. A) CtBP1 and **B)** CtBP2 across a panel of provisional TCGA tumor samples as in **Fig. 1_3**. RNA sequencing (RNAseq) was performed and run through the RNA Seq V2 pipeline. Expression data is displayed as log₂ values.

Chapter 2: Discovery of small molecule inhibitors through high-throughput screening and rational design

Rationale:

CtBPs play an integral role in cancer maintenance and progression by activating proto-oncogenes and repressing tumor suppressors. Inclusive with these findings, it has been reported that targeting CtBP for proteasomal degradation can lead to apoptosis independent of p53 status.^{57,76} Further evidence of a pro-oncogenic phenotype is seen in patient cohorts spanning different cancer types. These findings work together to provide an impetus for studying whether or not CtBPs can be therapeutically viable targets for cancer treatment.

There are a number of characteristics of CtBPs that make the family a good candidate for cancer therapeutic targeting.⁷⁷ For one, the basic mechanism of action of CtBP is understood: in the presence of NADH and MTOB, CtBPs can undergo an enzymatic redox reaction that oxidizes NADH to NAD⁺ and reduces MTOB to the compound 4-methylthio-2-hydroxybutyric acid, or MTHB. The understanding of this slow intrinsic enzymatic activity allows for the development of assays to look for small molecule inhibitors. By combining purified CtBP protein, an excess of NADH, and MTOB together, the redox reaction, as demonstrated by the conversion of NADH to NAD⁺, can be measured in a spectrophotometer. NADH absorbs light at 340 nanometers, while the oxidized NAD⁺ does not. This assay can be upscaled to perform high throughput screening of small molecular inhibitors. Relevant drugs will decrease the rate of enzymatic activity, and hits can be verified and tested for cytotoxicity and CtBP-specificity.

CtBPs also meet several other criteria distinctive of ideal drug targets.⁷⁷ We have discussed that CtBPs are upregulated in several different cancer types as compared to adjacent normal tissue, suggesting that it is, in fact, disease modifying. This also suggests that CtBP-

specific compounds *in vivo* would preferentially target tumor cells, with limited off target effects for normal tissue. Indeed, as discussed below, there is evidence that targeting of CtBP by the CtBP-substrate (and occasional inhibitor) MTOB is more toxic in malignant versus normal cells, providing a case that modulation of CtBP is less important under physiological conditions than in a cancer context. We show in this chapter that we have been able to visualize the druggability of CtBP through crystal structure and enzymatic assay. Given these observations, we claim that CtBP is a good target for cancer therapeutic development. Inclusive among these characteristics, the Grossman lab has previously demonstrated the relevance of CtBP targeting through studies with the substrate MTOB.

In 2010, the Grossman lab reasoned that since MTOB can inhibit CtBP function at high concentrations, it may be able to act as a CtBP-specific cancer therapeutic at high doses. In addition to several cell based assays, the lab worked to study the therapeutic efficacy of MTOB in mice⁷⁰. In order to study the safety of MTOB treatment *in vivo*, the Grossman lab injected mice with 750 mg/kg MTOB twice a week for four weeks. When compared to PBS injected controls, all major organs were indistinguishable between the two groups, and throughout the study no signs of illness or distress were observed. Interestingly, when mouse embryonic fibroblasts (MEFs) were treated with MTOB, the degree of cellular toxicity was directly related to the degree of cellular transformation. Incubating 4mM MTOB with non-transformed or “normal” MEFs, showed no difference in cell viability as compared to untreated samples. However, MEFs from p19-Arf null mice were more susceptible to apoptosis (65% survival reduction after 7 days). p19-Arf-null MEFs with c-Myc and activated Ras were even more vulnerable to 4mM MTOB treatment after 7 days (98% survival reduction). Therefore, there is evidence that the more highly mutated a tumor is, the more susceptible it may be to MTOB

treatment.⁷⁰ This targeting was seen in tumor development: *Nu/Nu* mice were injected with 3×10^6 HCT116 p53^{-/-} cells by IP injection. After 7 days, IP injections with PBS or 750mg/kg MTOB were administered thrice weekly for 8 weeks or until sacrifice was necessary due to tumor burden. MTOB treated mice showed a median tumor-free survival of 38 days versus 35 for PBS controls, but 48% of MTOB treated mice were tumor free at the time point when all PBS mice showed visible tumors. PBS treated mice had tumors weighing 4.7 grams on average, while MTOB treated mice had an average tumor size of 1.2 grams, with a higher degree of apoptosis (measured by TUNEL staining) than control mice.

Taken together, this shows CtBP-specific therapies may preferentially target cancer cell populations, and CtBPs are ideal inhibitors for drug development. The work done with MTOB provides evidence that further therapeutic development may yield dividends in treating cancers with high CtBP expression. However, MTOB must be used in high doses to surpass substrate level concentrations. As MTOB is an intermediate in the methionine salvage pathway, non-cytotoxic levels of MTOB may also participate in methionine recycling, leading to unintended consequences that may give cancer cells a proliferative advantage. Thus, it would be necessary to find a similar inhibitor to MTOB that can impede CtBP function at much lower levels, while eliminating the normal physiological processes attributed to MTOB.

Based on the positive results seen by these studies and the unique structure of the CtBP substrate-binding site, we set out to discover highly specific inhibitors of CtBP. In this chapter we vet potential inhibitors through a combination of high-throughput screens and rational design based on MTOB and crystal structure. From this work, we discover a compound, 2-hydroxy-3-imino-phenylpropionic acid (HIPP) that binds CtBP with more than 1000-fold greater affinity than MTOB and potently inhibits enzymatic function. We also propose a scheme in which CtBP

undergoes an ordered mechanism of formation requiring NADH binding prior to that of MTOB. HIPP is suggested to bind prior to NAD⁺ release, but after the redox reaction with MTOB, providing valuable insight into how inhibitors may disrupt CtBP-complex formation.

Materials and Methods:

Protein Purification:

An N-terminal truncated (a.a. 31-364) CtBP2 protein containing the functional dehydrogenase domain and an N-terminal his-tag was cloned into a pet-28a vector and used for enzymatic studies. The C-terminal 90 residues of CtBP2 are unstructured, and previous attempts to use full length CtBP2 were confounded due to the formation of protein aggregates, and so we opted for a truncated protein that retains dehydrogenase activity. CtBP2 was transformed into BL-21 De3-Ril⁺ cells and grown under kanamycin selection. After inoculation of large cultures, cells were grown to an OD₆₀₀ (Optical density at absorbance 600nm) of 0.5-0.6. Protein expression was induced by 200 μ M IPTG for 4 hours, and the culture was pelleted and homogenized. Cell membranes were discarded and the solute was incubated in a nickel bead solution for 90 minutes. The bead mixture was added to an equilibrated column and allowed to drain by gravity flow. After several washes, the purified protein was eluted in 300 mM Imidazole and dialyzed overnight. Purity was assessed by Coomassie staining. Proteins were stored in 50% glycerol for long-term storage. The recipes for each buffer are as follows:

Wash I: 300mM NaCl, 50mM Tris pH 7.3, 40mM Imidazole pH 7.5, 1mM DTT

Wash II: 300mM NaCl, 250mM Na-pyruvate, 50mM Tris pH 7.3, 40mM Imidazole pH 7.5, 1mM DTT

Wash III: 300mM NaCl, 50mM Tris pH 7.3, 40mM Imidazole pH 7.5, 1mM DTT, 0.5% Triton-X 100

Wash IV: 2M NaCl, 50mM Tris pH 7.3, 40mM Imidazole pH 7.5, 1mM DTT

Elution Buffer: 300mM NaCl, 50mM Tris pH 7.3, 300mM Imidazole pH 7.5, 1mM DTT

Dialysis Buffer: 300mM NaCl, 50mM Tris pH 7.3, 40mM Imidazole pH 7.5, 2mM DTT, 5% Glycerol

High Throughput Screen:

High throughput drug screening experiments were done in collaboration with the UMass Medical School Small Molecular Screening Facility. The Chembridge 30,000 compound Diverset was screened and Greiner bio-one UV-star 384 well plates were used due to their lack of interference at $A=340\text{nm}$. An NADH disappearance assay, was performed to measure dehydrogenase activity (the conversion of NADH to NAD⁺). Briefly, the assay is performed in a buffer consisting of 1mM DTT, 25mM HEPES, and 25 mM KCl. The first two columns of each plate contained positive controls; purified (a.a. 31-364) CtBP2, NADH, and substrate levels of MTOB are loaded with the addition of 1uL DMSO. The last two columns are negative controls, containing MTHB instead of MTOB, without the addition of drug. 60uL of premixed 100 μM NADH and 66.7 μM MTOB are added to columns 1-22 by μFill (Biotek), followed by the addition of 60uL premixed 100 μM NADH and 66.7 μM MTHB into columns 23 and 24. 1uL of 5mM drugs are then added from the drug screen plates (in 96-well format) into the assay plates by use of the TeMo robot (Tecan). Finally, 19uL of 84.4ug/mL purified CtBP2 is added into each well, yielding a total volume of 80uL per well. Absorbance was read immediately after adding protein with the Safire plate reader (Tecan). Absorbance was measured again at 1 hour and 2 hours post incubation. The final concentrations of reagents in the assay are 20.045 ug/mL CtBP2, 75 μM NADH, 50 μM MTOB/MTHB, and 62.5 μM drug.

Quality assessment is assured by looking at positive and negative controls at the 1-hour time point. After 1 hour the positive controls should show ~50% loss of absorbance and the

negative controls should show a decrease of less than 0.02 absorbance units. On average the positive controls should decrease in absorbance about 0.2 absorbance units with the negative controls showing 0.02 units of absorbance reduction. Hits are first identified as those compounds that show a loss in absorbance of less than 0.1. After the first round of screening, hits that show increased absorbance from the initial point were considered spurious results and removed from further assessment. The hits were arranged from highest to lowest loss of absorbance, and the 21 hits with the lowest change in absorbance were identified for use in follow up validation screens.

Compounds found from the initial screen were ordered from either Chembridge or Molport drug synthesis companies and were subjected to an NADH disappearance screening similar to that described above. In addition, three columns would contain negative controls: one without NADH, one without protein, and a column that replaces MTOB with MTHB. Except where noted, these controls contain all other components necessary for the reaction.

MTS Cell Viability Assay:

H1299 lung cancer cells were seeded in a 96 well plate and incubated overnight. The next morning drugs or DMSO control were added and cells were incubated for 72 hours. MTS assay was performed using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay according to the protocol provided by Promega.com. Briefly, 20uL of One Solution Reagent containing MTS tetrazolium was added to each well and the solution was further incubated 4 hours. Absorbance at A=490nm was read using the Biotek H1 plate reader. Statistical analysis was performed using Graphpad Prism®, by One Way Anova followed by Tukey's post-test.

Enzyme Kinetics for HIPP Ki Determination:

Purified CtBP2 (a.a. 31-364) in 50% glycerol was added to 150 μM NADH and variable amounts of MTOB and HIPP in a buffer containing 25mM HEPES, pH 7.1, 25mM Potassium Chloride, and 1mM DTT. The final concentration of CtBP was 40 $\mu\text{g/mL}$ (986 nM) per reaction. Solutions of HIPP in DMSO were added to account for 1% of the total volume. Final MTOB concentrations of 36, 24, 12, 8, 4, 2, 1, and 0 μM were used for 0 μM HIPP (1% DMSO control). MTOB concentrations for 500nM HIPP were: 64, 48, 24, 12, 8, 4, 2, and 0 μM . MTOB concentrations for 1 μM HIPP were: 80, 64, 48, 24, 12, 8, 4, and 0 μM . MTOB concentrations for 2 μM HIPP are: 96, 80, 64, 48, 36, 24, 12, and 0 μM . Components of the reaction were added to 96-well UV-Star Microplates (Greiner Bio-One). Upon addition of CtBP, reactions were mixed vigorously by pipetting and immediately read in the Synergy H1 microplate reader (BioTek). Absorbance was measured at $A=340\text{nm}$ every 7 seconds for 7 minutes to measure CtBP dehydrogenase function (NADH, but not NAD^+ absorbs light at 340nm). Assays were carried out at 25°C . The slope of the initial rate of the reaction was used to determine initial velocity of the reaction and model fitting, IC_{50} , and K_i were determined using Graphpad Prism (Version 5.04).

IC_{50} Determination for HIPP and PPy:

For PPy and HIPP IC_{50} determination, an NADH absorbance assay was performed as above, with an endpoint of 15 minutes. PPy concentrations were 400, 300, 200, 100, 10, 1, and 0.1 μM . Results reflect two independent experiments, each performed in triplicate. HIPP IC_{50} was plotted on a logarithmic scale with concentrations ranging from 10^{-4} to 10^2 μM concentrations of HIPP. Results represent three independent experiments, each performed in triplicate.

Results:

High throughput screening for novel CtBP-specific inhibitors

Following the discovery of MTOB as a CtBP-specific therapeutic reagent, the Grossman lab developed an assay that measured NADH consumption as part of CtBPs enzymatic activity. This process involves a redox reaction with MTOB. The assay, known as an NADH disappearance assay, relied on the preference of CtBP for NADH over NAD⁺ and the fact that NADH, but not NAD⁺ absorbs light at 340nm. Since CtBP has an intrinsically unstructured C-terminus,⁷⁸ and to prevent aggregation, we used a truncated version of CtBP for protein synthesis consisting of amino acids 31 to 364 (445 a.a. total). The Grossman lab chose to preferentially focus on CtBP2 over CtBP1, in hopes of seeing more striking differences. We conjectured that since CtBP2 has a nuclear localization signaling motif (NLS), absent in CtBP1, and that CtBP1-null mice are small and viable, but CtBP2-null mice are embryonic lethal, targeting of CtBP2 would be a more effective target for drug development, and provide more biological information in cell systems. We further suggest that since CtBP1 and CtBP2 have redundancy in function and structure, discoveries in CtBP2 will translate to being similarly relevant to CtBP1. As such, all experiments presented in this paper were performed with CtBP2.

Buoyed by the success of MTOB as a drug therapy, we conjectured that we could find other novel inhibitors that disrupt CtBP binding. As a first step, we performed a high throughput screen. With the help of Dr. Hong Cao at the University of Massachusetts Medical School, we tested 30,000 molecules using the Chembridge® Diverset on a modified NADH disappearance assay. We tested final drug concentrations of 62.5 μ M and calculated the change in absorbance ($A=340\text{nm}$) 2 hours after the addition of CtBP2 protein to a buffer which contained drug, 75 μ M NADH, and 50 μ M MTOB final concentrations. Wells containing the reduced form of MTOB,

MTHB were used as negative controls. Drugs that visibly distorted the pigment of the reaction mixture or that showed an increase in absorbance were excluded from analysis. The remaining compounds were scanned and ordered from the least to most change in absorbance. The top 100 compounds were identified and eight of the lead compounds were ordered for validation studies.

Each of the top 21 hits demonstrated a total loss of absorbance of less than 0.05 absorbance units after 2 hours, compared to 0.02 units with our negative controls and 0.2 units with no-drug controls. With the aid of Dr. Keith Ellis in the VCU Department of Medicinal Chemistry we were able to postulate the druggable moieties of MTOB that can be altered to create potential inhibitors. Assessing our top hits showed that of 21 compounds, 11 fell into 3 different groups (**Fig. 2_1**). It is possible that the conserved thiourea moiety in group 1 (**Fig. 2_1A**) will function as an early transition state mimetic for the ketone substituent in MTOB and bind within the substrate-binding domain of CtBP. It was believed that Group 2 molecules may function in NADH domain binding, rather than substrate binding. Group 3 exhibits interesting electronics and may shift electron density within the binding domain. Each of the compounds ordered for validation come from groups 1 and 2.

Follow up experiments were performed to verify the accuracy of our results and to test for cytotoxicity. Using an H1299 lung cancer cell line, an MTS assay was performed to measure cell viability after 72 hours of drug treatment (**Fig 2_1B**). The results were compared to viability observed in a DMSO-only control and a new compound, 2-hydroxy-3-imino-phenylpropionic acid, or HIPP (discussed below). Each compound showed significant cytotoxicity compared to control, with the transition-state mimetic-like drug, compound 1, showing approximately 50% decrease in viability at 1mM concentration. The putative NADH-domain mutant, compound 8, showed roughly 50% cytotoxicity at 50 μ M concentration, with cell cytotoxicity increasing with

drug concentration. While these compounds seemed suggestive of good targets in cancer viability assays, they failed validation by repeat NADH disappearance assays. Several of the new compounds, in addition to a rationally designed molecule, phenylpyruvate (discussed in the subsequent section), were tested across a logarithmic range of values ranging from 5nM to 500 μ M concentration for 1 hour. Results are shown in **Figure 2_1,C-D**. None of the compounds tested showed rates of inhibition that were comparable to the initial screen results. At 500 μ M drug concentration, compound 4 seemed to increase absorbance, likely due to absorbance of the compound itself. Compounds 5 and 7 at 500 μ M concentration do show apparent significant inhibition. However, it should be noted that this concentration far exceeds that of both MTOB and NADH concentrations, and may not represent a true inhibitory reaction.

While the data did not identify a new class of inhibitors, study of Group 1 compounds gave birth to ideas that could be incorporated into rational design; specifically, that transition state mimetics could provide a scaffold for inhibitor design. Concurrently with these results, colleagues Dr. William Royer and Dr. Brendan Hilbert at the UMass Medical Center were able to determine the crystal structure of MTOB binding to CtBP1 and CtBP2.⁷⁹ This finding helped to distinguish key residues of the substrate-binding domain pocket. Within this domain, they found that CtBP1 and CtBP2 active sites were nearly identical. These involved tight and stoichiometrically favorable interactions of MTOB with two arginine residues, a tryptophan, and a histidine in the active site (**Fig. 2_2A**). In particular, they observed the tryptophan residue (Trp318/324 in CtBP1/2 respectively) interaction with the MTOB thiol moiety of CtBP1 to be interesting. A BLAST search revealed this tryptophan to be exclusive to CtBP family proteins; a variety of other amino acid substitutions are found in otherwise similar dehydrogenases. Previous work with CtBP crystal structures demonstrated a tight interaction between this

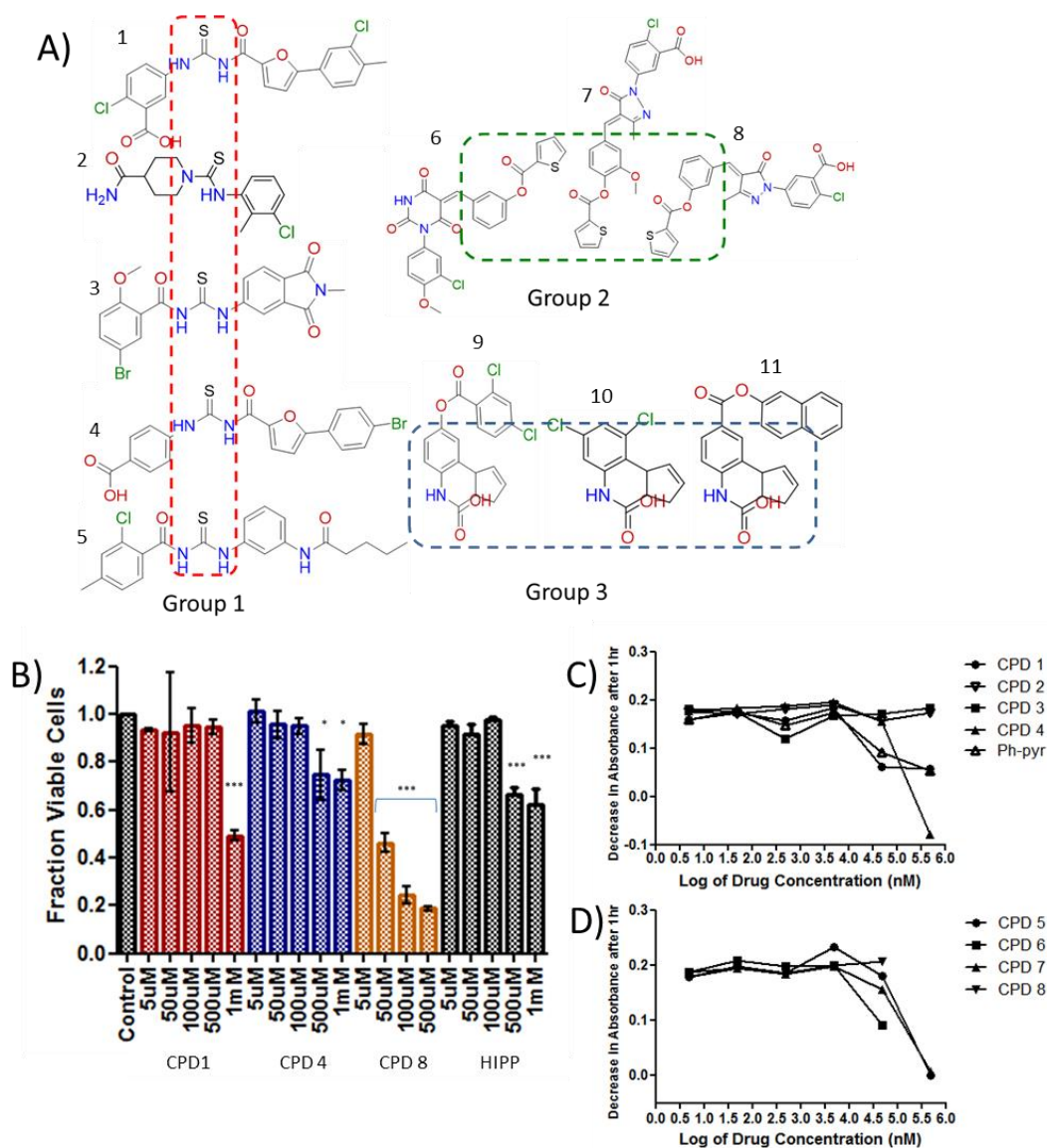


Figure 2_1. Discovery and validation of potential therapeutics by high-throughput screen. 11 of the top 21 hits from a 30,000 compound screen contained common moieties. **A)** shows the three groups with common residues outlined. **B)** MTS viability assay for compounds (corresponding to numbers in **A)** from groups 1 and 2, along with 2-hydroxy-3-imino-phenylpropionic acid, HIPP. * = p-value < 0.05, *** = p-value < 0.001. **C)** and **D)** NADH disappearance assay for compounds 1-8 and phenylpyruvate, with concentrations of 0, 5, 50, 500nM, and 5, 50, and 500µM. Values plotted on a logarithmic scale.

tryptophan with bound NAD⁺,³⁹ and other studies have suggested the residue may play a role in oligomerization.⁴⁴ Together this suggests that the unique substrate-domain tryptophan may operate in bringing substrate and NADH co-factor in close proximity for enzymatic function. Further, having a unique residue that exists in only the CtBP family of molecules provides a target for designing specific inhibitors that may have limited off-target effects.

Discovery of HIPP as a CtBP2 inhibitor

Taken together, the knowledge from crystallographic data and the inspiration gleaned from high throughput screening allowed for rational design of inhibitors. This was done with the help of Dr. Keith Ellis and associates. Efforts were employed to use the basic structure of MTOB as a ‘backbone’ to create a better therapeutic. Since the CtBP2 tryptophan, W324, residue may function as a sort of keystone, being integral for dimerization, dehydrogenase function, and oligomerization properties of the protein, we first sought to enhance the binding capacity of the MTOB substrate by replacing the thiol group with a phenyl ring that would allow for pi-pi interactions with the catalytic tryptophan (**Fig. 2_2B**). This substitution forms phenylpyruvate (PPy). Phenylpyruvate showed a slight increase in inhibition potential, but interestingly gave cells a proliferative advantage when added to media (**Fig. 2_2C**). While the mechanism for this has not been investigated, phenylpyruvate is a bi-product of altered phenylalanine processing in patients that have the metabolic disorder, phenylketonuria, a disorder characterized by an inability to process phenylalanine.⁸⁰ It is possible that this molecule is able to be recycled within cells to provide metabolites that bestow a growth advantage.

It was then hypothesized that we could further increase inhibition potential by altering moieties within the α -ketone of phenylpyruvate (**Fig. 2_2B**). 2-hydroxyimino-3-phenylpropionic acid (HIPP) replaced the phenylpyruvate α -ketone with an oxime in an attempt to create a

transition-state mimetic of the redox reaction between the carbonyl and NADH, without allowing for actual hydride (H⁻) transfer. Analysis of HIPP, along with phenylpyruvate, were tackled on two fronts. First, Drs. Royer and Hilbert determined the HIPP/CtBP/NAD co-crystal structure and performed ITC experiments to determine the K_d of binding, among other analyses for each of these molecules, comparing them to MTOB. Second, we performed enzymatic experiments to obtain information about its functional inhibition potential.

Crystallographic results of MTOB, phenylpyruvate, and HIPP bound to CtBP1 in the presence of nicotinamide adenine dinucleotide (NAD⁺) are shown in **Figure 2_3,A-C**.⁸¹ The structures show both hydrogen bonding and coulombic interactions, a measure of affinity based on electronic charge. Several observations were made; foremost is that binding of each of these small molecules does not substantially change the substrate-binding cleft, with exception to a shift in A123 in the canonical binding of phenylpyruvate. This amino acid is part of a hinge that brings the substrate binding domain and NAD-binding domain within close proximity.⁷⁹ As we had predicted, the phenyl group of Trp318 stacked tightly with the phenyl groups of both phenylpyruvate and HIPP. They showed approximately 2-fold tighter binding with this residue than did MTOB (measured by van der Waal's contacts). However, the general conformations were somewhat unexpected. MTOB binds in a canonical conformation, one in which the ketone on carbon C-2 is brought close in proximity to NADH. This allows an interaction that permits a redox reaction to occur, converting MTOB to MTHB and NADH to NAD⁺. Phenylpyruvate assumes this same conformation with its C-2 ketone roughly half the time (**Fig. 2_3B**), and a non-canonical conformation (**Fig. 2_3C**) the other half the time that phenylpyruvate binds. The non-canonical conformation forces the molecule to pivot such that C-2 points away from NADH,

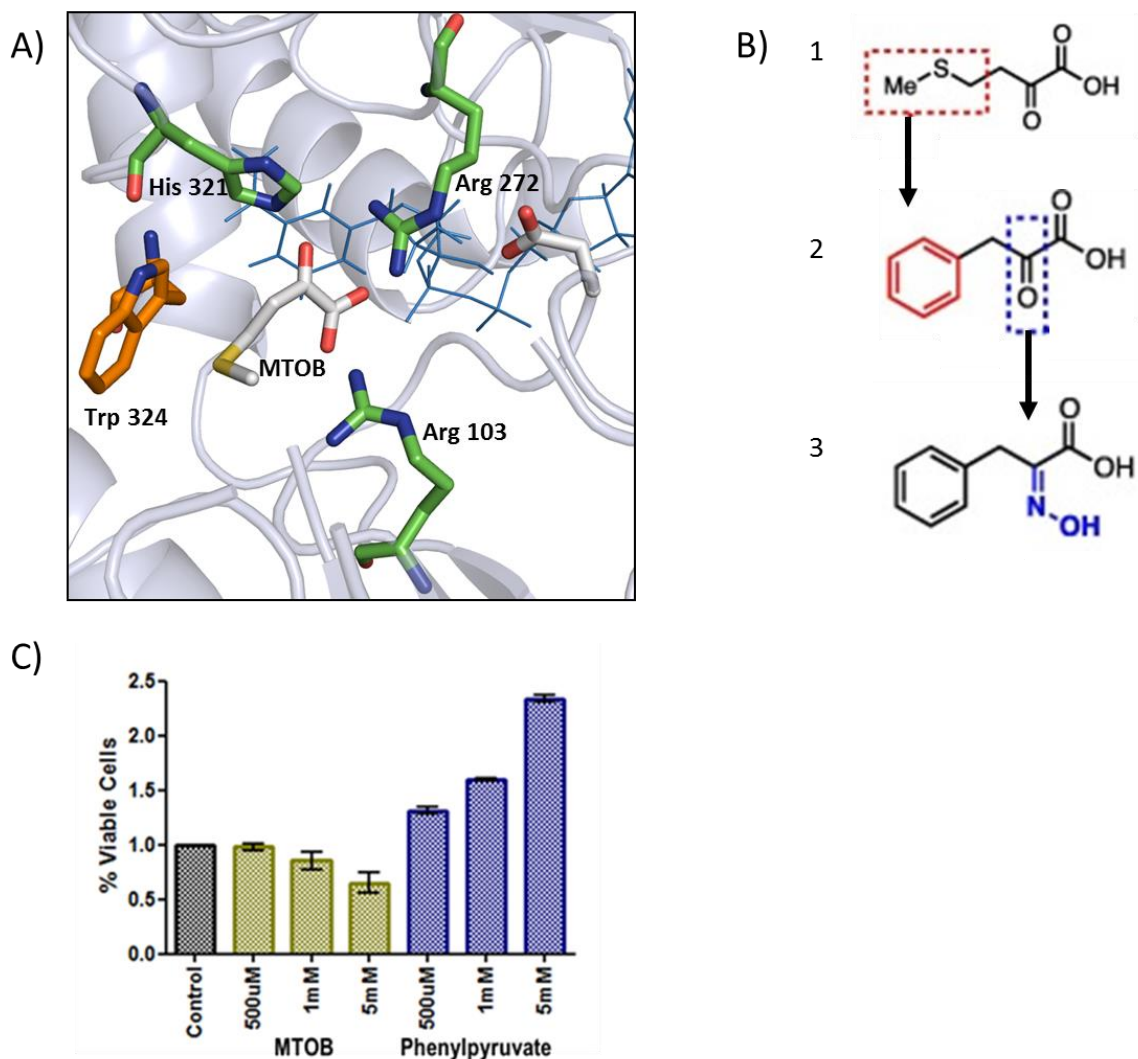


Figure 2_2. Crystal structure of substrate-bound CtBP provides insight into rational design. **A)** CtBP1 binding MTOB in the presence of NADH (blue skeleton). Shown are key residues of the Substrate binding domain. Blue segments indicate nitrogen bonds, Red – oxygen, and yellow – sulfur. H321, R103, and R272 are common among similar D2-dehydrogenases. **B)** Rationale for deriving novel CtBP-specific inhibitors. **1** – The thiol of MTOB was replaced with a benzene ring to form PPy – **2**. The oxime was substituted with a hydroxyimino group to form HIPP – **3**. **C)** MTS cell viability assay performed with H1299 lung cancer cell lines. While PPy has enhanced pi-pi interaction, it does not increase cytotoxicity within cells, but rather provides a proliferative advantage.

fills part of the active site, and can no longer participate in hydride transfer. HIPP surprisingly binds only in the non-canonical conformation (**Fig. 2_3D**). This is surprising because our hypothesis was that the oxime group on C-2 of HIPP would interact with NADH, but would force inhibition by preventing an actual redox reaction from occurring. It is believed that a canonical conformation for HIPP would cause clashing interactions with a unit of the substrate binding domain, H315 (**Fig. 2_3D**).

A unique feature of the CtBP active site is that it is largely hydrophilic and contains a water network within the substrate-binding cleft⁷⁹. Phenylpyruvate had a shift in the hinge structure that caused displacement of two of the four water molecules found near MTOB (**Fig. 2_3,E-F**). HIPP also, in effect, displaced two water molecules, but did so without displacing the hinge structure, similarly to that seen with phenylpyruvate (**Fig. 2_3G**). This may be useful in the design of future inhibitors, as the effect on hinge displacement has not been evaluated, and may have disruptive repercussions. It may also be useful with future HIPP-like inhibitors to aim to more fully displace other water molecules.

Isothermal calorimetry experiments (ITC) using a competitive displacement setup served to demonstrate the differences in affinity for CtBP between MTOB and HIPP. For this assay, MTOB was incubated with purified protein, and then HIPP was titrated into the reaction to displace MTOB from the active site and ITC measurements were obtained. The dissociation constant, K_d , which in this case measures the ability of the molecules to fall off CtBP, was then calculated. It was shown that MTOB had a $K_d=2.96 \pm 0.82$ mM. HIPP proved to have a 1000-fold higher affinity for CtBP2, with a $K_d=1.30 \pm 0.07$ μ M. This suggests that HIPP is a better inhibitor than MTOB by three orders of magnitude.

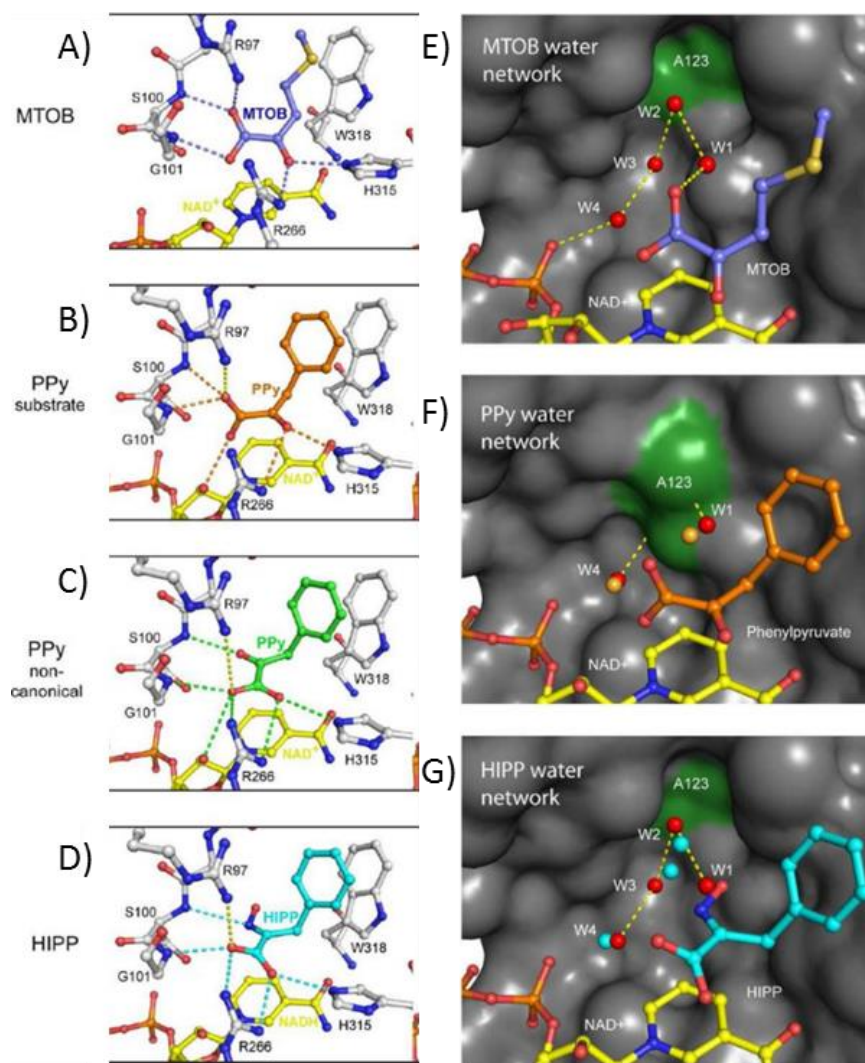


Figure 2_3. MTOB, PPy, and HIPP hydrogen residue interactions and water networks **A)** The hydrogen bond network (dashes) of substrate MTOB. PPy and HIPP are compared to this structure. **B)** The PPy substrate conformation possesses a similar hydrogen bond network to MTOB (orange dashes). This conformation has lost the hydrogen bond to R97, although PPy maintains proximity for coulombic interactions (yellow dashes). The substrate PPy has an additional hydrogen bond to NAD⁺. **C)** The noncanonical phenylpyruvate conformation's hydrogen network (green dashes). Orientation of the carboxylate towards R266 maximizes hydrogen bond potential as well as coulombic interactions (yellow dashes) with R97. **D)** HIPP forms a similar hydrogen bonding network (cyan dashes) and coulombic interactions (yellow dashes) to the non-canonical PPy conformation, with exception of losing the interaction with NAD⁺. **E)** MTOB possesses a water network (red spheres with yellow dashes) that connect the substrate to an NAD⁺ phosphate via four water molecules (W1–W4). The conformation of hinge residue A123 (dark green) helps create a cavity for the water molecules, a feature unique to CtBP. **F)** The water network (orange spheres) in the PPy structure is disrupted by the novel conformation of A123. W2 and W3 are completely displaced as the cavity collapses. W1 is present with the substrate conformation of PPy and when no molecule is bound in the active site (~60% of the time). **G)** The HIPP structure water network is altered compared to MTOB. W1 is completely displaced by the HIPP hydroxyl group. W2 shifted position to interact with HIPP. Due to shifts in both W2 and W3 (now 1.9Å apart), their presence is mutually exclusive. Similar to PPy, HIPP has no effect on W4 position.

Kinetic inhibition assays were performed to assess the ability of HIPP to inhibit CtBP catalytic activity. Inhibition assays measured CtBP dependent NADH oxidation in the presence of the substrate, MTOB, and inhibitor. Total change in absorbance ($A=340\text{nm}$) was measured after 15 minutes, using concentrations of drugs from 10nM to $100\mu\text{M}$ on a logarithmic scale. Consistent with our crystallographic results, both phenylpyruvate and HIPP substantially inhibit CtBP dehydrogenase function, exhibiting IC_{50} values of $135.9\mu\text{M}$ and 745.9nM , respectively (**Fig. 2_4,A-B**). 50% inhibition levels, or IC_{50} values, vary from one assay to another and are altered with any change in condition. They serve as a good way to compare two different conditions, but only in well designed and controlled assays that are identical in procedure. However, they fail to demonstrate a true constant value for an inhibitor. In order to determine this value, one must determine an inhibitor constant, or K_i .

In order to determine K_i measurements for a drug, one must determine the initial velocity of enzyme activity at several concentrations of drug and in the presence of several concentrations of substrate. For the purpose of this study, standard Michaelis-Menten kinetics were simulated. With this type of kinetics, one must ensure that there is a single substrate reaction such that $\text{E} + \text{S} \rightleftharpoons \text{ES} \rightarrow \text{P}$, where 'E' represents the enzyme, CtBP2, 'S' represents the substrate MTOB, 'ES' is the formed complex of these two molecules, and 'P' represents the product, production of MTHB from the redox reaction between MTOB and NADH. In order to mimic this single substrate reaction, it is necessary to keep NADH in far excess, so that it cannot play the role of a limiting factor. This set up is known as a pseudo-first order reaction. In this experiment there was, at the least, a $64\mu\text{M}$ excess of NADH in the system.

It is also necessary to determine the initial velocity of each reaction. The formation of an enzyme-substrate reaction is reversible, and once the interaction reaches equilibrium, the reverse

reaction, showing that enzyme-substrate complex disassembly, cannot properly be subtracted from the forward rate. This confounds results in determining the true rate of enzyme substrate assembly. At the beginning of the reaction, however, enzyme and substrate largely go toward product, giving a better estimate of the rate of the forward reaction. As such, the change in absorbance was measured within the first couple of minutes of the reaction to find this initial velocity. K_i was determined for HIPP. A more thorough analysis using K_i was not possible with phenylpyruvate, as it demonstrated substrate activity at low concentrations.

One of the key benefits of determining K_i in a uni-substrate system is that it can confirm the mode of inhibition. Using Michaelis-Menten kinetics, one can infer whether the inhibition occurs through competitive or non-competitive means. Competitive inhibitors bind in the same site as a substrate. Based on the crystal structure of CtBP bound to both MTOB and HIPP, we know that they compete for the same active site. A non-competitive inhibitor would suggest that the inhibitor binds at another location outside the active site, but that this binding causes a change, conformational or otherwise, in the enzyme that prevents the substrate from binding its active site. We have been able to see in our crystal system that non-competitive inhibition does not occur. These two modes of inhibition can be observed in Michaelis-Menten kinetics through changes in the maximum velocity that can be achieved by the enzyme in the presence of inhibitor, V_{max} , or through a change in the substrate concentration that is required to see half the maximum velocity, K_m . A change in K_m is associated with a competitive inhibitor and a change in V_{max} is associated with that of a non-competitive inhibitor.

HIPP, however, was found to have a K_i of 928nM through an apparent non-competitive mechanism ($R^2= 0.7556$; **Fig 2_4C**). This contrasts to a competitive mode of inhibition for HIPP, which had worse model fitting ($K_i=109.1nM$; $R^2=0.4995$). This can be visualized by

noting the decrease in maximum reported velocity as inhibitor is added (**Fig 2_4C**) or in a double reciprocal plot (**Fig 2_4D**). A competitive inhibitor in this plot, known as a Lineweaver-Burk plot, shows lines that intersect along the y-axis, whereas a non-competitive inhibitor intersects along the x-axis. Based on model fitting, represented by comparison of R^2 values, the non-competitive model is a better fit. In addition, the validity of a K_i value can be strengthened by comparison with ITC data. An accurate K_i should fall within 2-fold of the range of the K_d generated from ITC. Our K_d for HIPP bound enzyme was $K_d=1.30 \pm 0.07 \mu\text{M}$, which points toward the non-competitive like model. From model fitting and the observation that the K_d from ITC is similar to that of the K_i from the non-competitive model we do not have a basis to reject our data outright.

The result was contrary to the hypothesis inferred from our crystal data. To resolve this perceived paradox, we looked to alternative interpretations. An investigation of the literature revealed that allosteric interactions may be responsible for our result. An allosteric reaction involves two or more ligands wherein one ligand affects the binding of another⁸². In 1964, a variation on standard notions of allosteric regulation was identified⁸³. This concept, known as V-type allosteric systems describes a system wherein there are two ligands and an enzyme. One ligand can bind independently of the enzyme-state or the presence of the other ligand. The second ligand, however, is dependent on the first. This differs from standard ideas of allosteric systems, because typically in a two ligand system, both ligands will affect and be affected by the binding capacity of one another. Oftentimes a V-type system will look like a non-competitive inhibitor whenever there is excess of a substrate (in our system, MTOB) that can bind independently of the status of the other ligand in the reaction. V-type systems also can show a

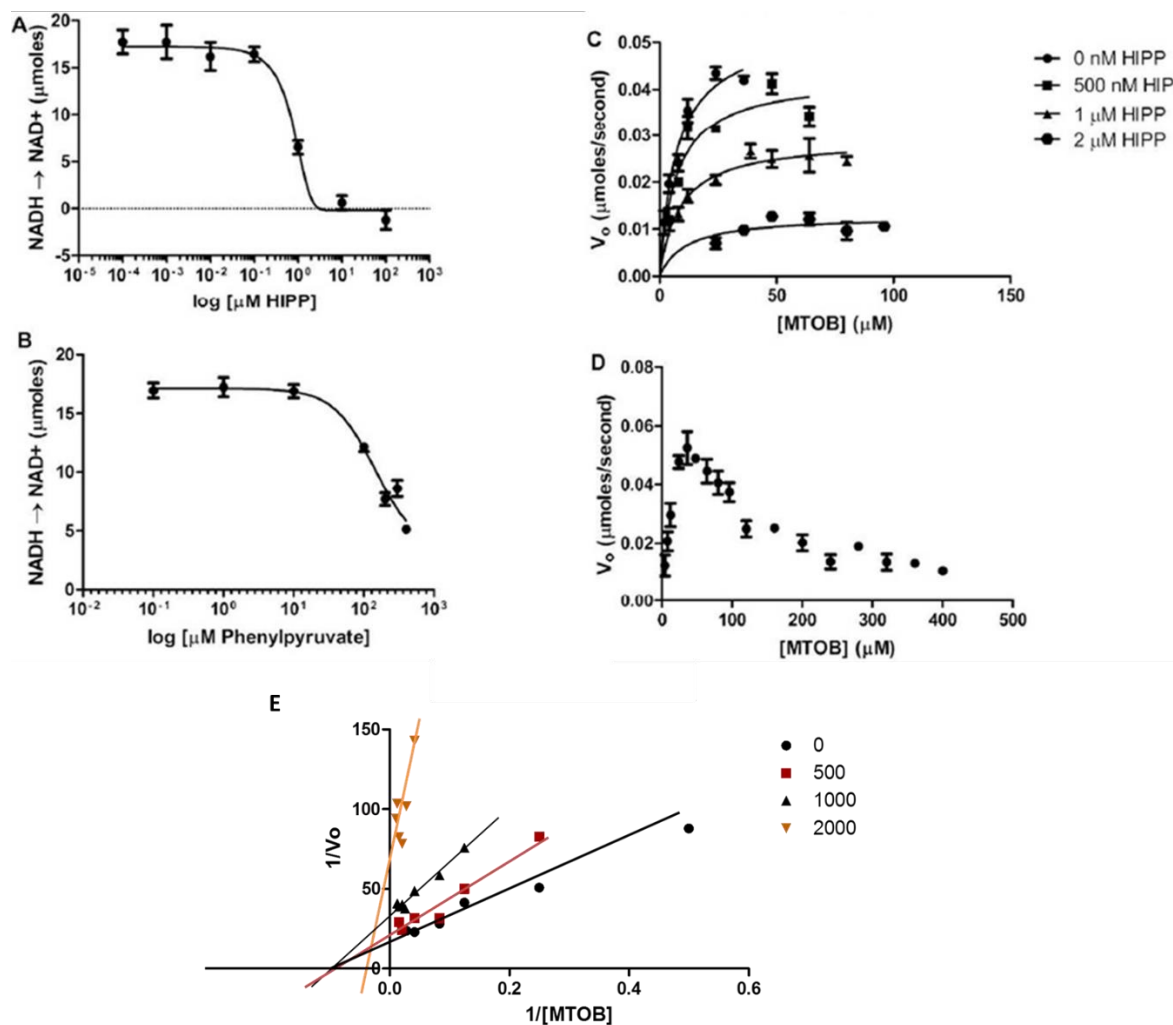


Figure 2_4. CtBP2 enzymatic inhibition assays. IC₅₀ measurements for HIPP, **A**), and PPy, **B**), after 15 minutes show that HIPP exhibits an IC₅₀ value more than 100 fold lower than PPy. Data represent n=3 and n=2 triplicate experiments, respectively. K_i plots, **C**) demonstrate that HIPP inhibition results largely from a decrease in V_{max}, which would not be expected for a purely competitive inhibitor. The plateau of each hyperbolic curve illustrates V_{max} for individual drug concentrations. Points represent the average of n=7 reads. MTOB, **D**) exhibits substrate inhibition when in excess. n=2 independent triplicate experiments. All error bars represent standard deviation. **E**) Lineweaver-Burk plot showing a double reciprocal transformation of the data presented in **B**. This and other figures reprinted with permission from Hilbert BJ, Morris BL, Ellis KC, et al. Structure-guided design of a high affinity inhibitor to human CtBP. *ACS Chem Biol.* 2015;10(4):1118-1127. doi: 10.1021/cb500820b Copyright 2015 American Chemical Society

non-competitive like model, whenever there is an inhibitor that competes for that same active site.⁸⁴

Similar to this concept, many bi-substrate enzymes, including another D2-dehydrogenase, D-2-hydroxy-4-methylvalerate dehydrogenase, has demonstrated abortive ternary complexes.⁸⁵ It has been shown that D-2-hydroxy-4-methylvalerate dehydrogenase undergoes a rapid equilibrium ordered bi-bi ternary complex mechanism, with NAD binding first, followed by the substrate. Addition of an inhibitor caused the complex to be bound in a state where the substrate was released, but inhibitor bound before the release of the coenzyme product. They were also able to see substrate inhibition when substrate exceeded stoichiometric levels of NAD. In another instance, using a pseudo-first order reaction, it was shown that phosphoglycerate dehydrogenase similarly had a loss in V_{max} , suggestive of a V-type allosteric system.⁸⁶ D-3-phosphoglycerate was shown to have a hinge domain that closed around NADH whenever it was bound. Serine acted as an inhibitor and was capable of preventing closure of the hinge domain.⁸⁷

CtBP meets many of the criteria suggestive of a similar mechanism of action. In addition to a loss of V_{max} in our K_i inhibitory studies, CtBP has been shown to similarly have an open and closed conformation based upon the presence of NADH.^{39,88} When NADH binds, this closure induces a change in conformation that permits dimerization. Also, MTOB overexpression causes substrate inhibition (**Fig 2_4D**).

Taken together, the hypothesis for CtBP's mechanism of action, as accumulated from both our individual work and the compilation of literature pertaining to similar D2-HDH dehydrogenases is as follows: CtBP, in the presence of high amounts of NADH, binds the coenzyme NADH in an ordered bi-substrate reaction. It undergoes a physiological change, which causes a closed conformation of the enzyme. The closed conformation allows for

dimerization and transcriptional regulation. When MTOB or other substrate binds, a redox reaction occurs, leading to recycling of the enzyme. However, when MTOB is in excess of NADH, MTOB can bind first, disregarding the ordered mechanism, and preventing NADH from coming in and closing the hinge. This substrate inhibition prevents CtBP from performing its transcriptional functions. In the presence of the inhibitor HIPP, inhibition could occur at several points. HIPP can either bind directly to the empty enzyme, or NADH and MTOB can undergo their typical reaction. It is believed that since NADH is enclosed within the CtBP holoenzyme, MTHB will be released prior to the release of NAD⁺. At this point, similar to many other bi-substrate enzymes, HIPP can bind before domain opening and NAD⁺ release. In this way HIPP may be promoting a ternary abortive complex. Dr. William Royer was able to derive simulations of HIPP bound to free CtBP enzyme along with HIPP bound in a ternary complex. He found a remarkable amount of concordance between his abortive ternary simulation and our data, involving a change in V_{max} with increased inhibitor concentration and including substrate inhibition. This simulation data, with a brief discussion of its derivation, is found in **Appendix I**. A visual representation of the possible mechanism for CtBP enzymatic formation and inhibition is detailed in **Figure 2_5**.

Together we have been able to find a next generation inhibitor with affinity for CtBP at 928nM. This was done by rational design, bringing together information obtained from both a high-throughput screen and crystallography data of MTOB-bound CtBP. Additionally, we have developed a hypothesis that can explain both HIPP's mechanism of action and the order of assembly of CtBP complexes. This research has provided a good deal of information in tying together seams of the CtBP story and gives some important insights into CtBP-targeting through

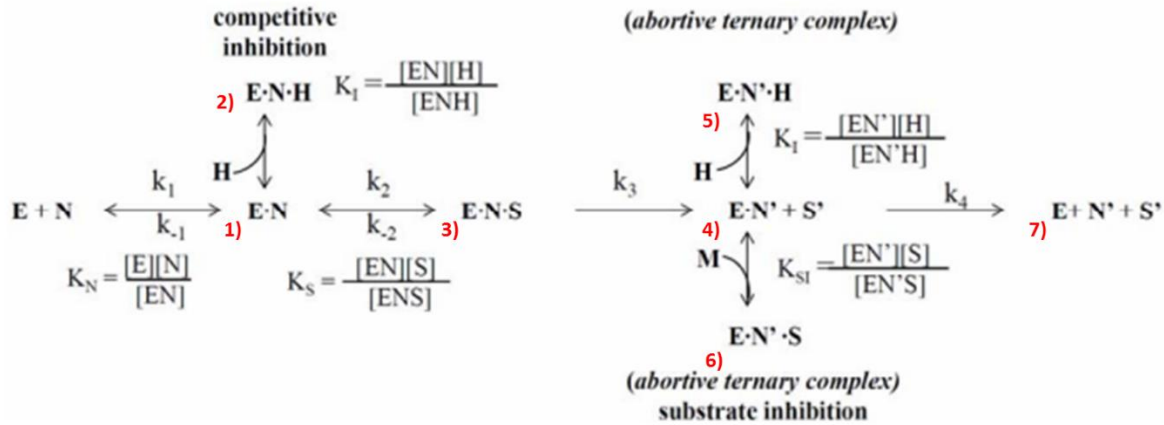


Figure 2_5. The hypothesis for CtBP inhibition of HIPP through an abortive ternary complex. In this schematic E= Enzyme, CtBP; N=NADH; S=Substrate, MTOB; H=HIPP (inhibitor); N'=NAD+; S' = MTHB; K= reaction rate. Each of the steps in this reaction are reversible, and the derivation of the rates of formation of each complex are shown. Briefly, following the schematic: Enzyme, CtBP binds NADH (1). At this point either HIPP (2) or MTOB (3) can bind. With MTOB binding, a redox reaction occurs, wherein NADH is oxidized to NAD+ and MTOB is reduced to MTHB (4). At this point, three possible things could happen: HIPP could bind to inhibit the complex (5), MTOB could bind to inhibit the complex (6), or NAD+ can be released (7) allowing for CtBP to be free to catalyze another reaction. Since NADH is found in excess in our system, it is presumed that most of the reaction goes through step 7, unless inhibitor is introduced. MTOB ternary inhibition likely only plays a small role since the substrate has a >1000-fold lower affinity than HIPP. In living cancer systems, where NADH is presumed to be in excess, it is believed that CtBP exists largely in state (1).

small molecular inhibitors. It also raises a number of important questions that should be addressed in the process of developing CtBP-based cancer therapies.

Discussion and Future Directions:

High throughput screening and fragment based drug design

We have been able to use insights gleaned from a high-throughput screen and crystal structure data of the substrate, MTOB, bound to CtBP to find a new and more potent inhibitor: HIPP. This compound consists of a phenyl ring that allows pi-pi stacking with a tryptophan residue within the substrate-binding domain that is unique to CtBP family proteins. It also contains an imine moiety that was found to more fully occupy the active site. The end result is a molecule that is over 1000-fold more potent at binding to CtBP than its predecessor, MTOB. Additionally, we hypothesize that HIPP causes an abortive ternary complex to inhibit the enzymatic function of CtBP.

With the experiments performed, there are limitations and several new questions to consider. For example, it is instantly perplexing that none of the tested compounds from our high-throughput screen were validated in follow up enzymatic assays. It may be impossible to know the reasons for this, but it is interesting to note that many of the potential hits segregated into families with common moieties. This would suggest that, in fact, the compounds were accurately identified, or rather that there was no misidentification with mapping the wells to their proper identification number in the database used. It is further possible that the compounds synthesized for inclusion in the high-throughput screen underwent different purification or synthesis methodologies than what was purchased for follow up experiments, and that impurities in one batch accounted for differences in binding. This option also seems unlikely since of the

two main groupings that we tested, which were based on common moieties, all showed similar binding patterns within their family. It is unlikely a preparation difference that was capable of fundamentally changing enzymatic interaction would be consistently observed across several separate molecules.

We worked to eliminate compounds from analysis that were not visibly transparent. Those compounds that significantly changed the color or clarity of the reaction were marked and noted for exclusion in the analysis. However, the potential exists that some of our families of compounds did in fact absorb light at 340nm, masking any reduction that could be caused by the NADH assay itself, but again, this was not observed in our follow up studies. Other possibilities imply more human error, like the chance that follow-up assays were performed incorrectly, or that a true inhibitor that was identified in our screen was not chosen for future study simply because we made the decision to only pursue compounds that segregated into groups of common moieties. Thankfully, despite the lack of true informative data, we did gain benefit from the screen. We were able to use the information from the group that contained a common thiol moiety to eventually develop an inhibitor that appears much more potent than MTOB. The effort served as a catalyst for rational design of drug targets, instead of a roadblock that impeded further small molecule drug discovery for CtBP.

We were able to use the structure of MTOB to identify a more potent inhibitor of CtBP enzymatic function, HIPP. While this rational design approach proved to be effective, it is possible that other design paradigms can be developed to target CtBP activity. Fragment based drug design (FBDD) provides an alternative approach to high-throughput screening that can inform new molecular scaffold designs for inhibitors.^{89,90} High throughput screening has been shown to be susceptible to providing false-positive hits. In many of these cases, this is due to

aggregation, where the drug molecule can sequester the protein of interest without actually interacting in a biochemically or physiologically relevant way. Also problematic is the molecular complexity of compounds in high throughput screens. Basically, the larger the molecule being studied, the more opportunities that molecule has to interact with the protein of interest. These interactions often may be non-specific to the function of interest, and may even involve unfavorable reactions. For example, a phenyl group of a molecule may increase inhibition potential, but an ester group on the same molecule may counteract or decrease binding affinity. In order to identify specific components that are important for inhibition, fragment based drug design has proved increasingly effective. This concept involves finding individual components that can interact with the protein, and then using these individual hits to assemble groups of molecules that contain several key moieties, with the hope that each component contributes additively to the inhibition potential.

FBDD involves its own considerations. A fragment is likely to bind CtBP protein more weakly than good molecular inhibitors with several components. As such, one must assure that the assay is sufficiently sensitive. The NADH disappearance assay used in the high throughput screen may be sufficient to detect useful components at high fragment concentration, but it is generally believed to be less sensitive than other techniques. One of the most commonly used techniques in FBDD is that of structure-activity relationship by nuclear magnetic resonance (SAR by NMR). Nuclear magnetic resonance allows observation of chemical shifts in the protein by addition of fragments. It can also identify the location of ligand binding among inhibitory components (the structure-activity relationship). Unfortunately, the screen requires large amounts of radioactive isotope labelled protein. The other commonly used technique with fragment screening is surface plasmon resonance (SPR). SPR involves immobilizing the protein

to a sensor chip and then running different fragments across the system. When a fragment binds the protein, it increases the mass near the surface of the sensor. One concern with SPR is the false discovery of nonspecific inhibitors. To distinguish these from true inhibitors, SPR experiments are often replicated with protein that involves mutations in the active site. Nonspecific inhibitors should bind nearly equally under these circumstances and can be removed from further validation. However, it is difficult to distinguish nonspecific inhibitors from allosteric ligands. The Grossman lab has generated several active-site mutants of CtBP (see **Chapter 4**), and the CtBP protein seems to be relatively stable, making SPR experiments an attractive alternative approach for future drug design.

FBDD is a viable possibility for rational design of inhibitors. We have developed tools that could aid in testing fragment based screening. In the pursuit of better inhibitors, the Grossman lab, in collaboration with Drs. William Royer, Keith Ellis, and Glen Kellogg, have developed docking simulations using the crystal structure of MTOB and HIPP-bound CtBP to predict good compounds for enzymatic and biological screening (further mentioned in **Chapter 3**). This, along with our NADH disappearance assay, may serve as a simple way to assemble novel CtBP-inhibitors using a fragment based approach to drug design.

Understanding the impact of HIPP inhibition on CtBP oncogenic function

Among the many questions raised from this study, perhaps one of the most pertinent is: what are the implications of HIPP's interaction with CtBP in a therapeutic context? Specifically, an issue is presented in using the enzymatic function of CtBP as a model to test drug compounds for utility against CtBP. One way in which an ideal drug target would function is to disassemble the CtBP2 complex, allowing for relieved expression of pro-apoptotic genes and other tumor

suppressors. Enzymatic disruption does not mirror disruption of an *in vivo* transcriptional complex. In fact, it is believed that CtBP's enzymatic function is a relic of its dehydrogenase lineage⁴⁶, and so disruption of CtBP enzymatic activity of itself does not prove that a drug will be therapeutically relevant. Rather, inhibitors can disrupt enzymatic reactions at several steps. An inhibitor could bind to free enzyme, to an enzyme/coenzyme (NADH) complex, or, following the loss of the reduced form of the substrate, it could bind prior to the release of the oxidized coenzyme. It is important to determine whether drugs in reality disassemble the complex. If drugs bind to a ternary complex, they may have undesired consequences, namely by providing a proliferative advantage to cancer environments by preventing further disassembly of CtBP oligomers that are complexed to target genes. Similar molecules, and our own simulation data, suggested that the predominant mechanism of HIPP action was on an abortive ternary complex. In truth, it is likely that more than one mechanism plays a role. The design of the NADH kinetic assay favored this mechanism, as NADH was held in high excess, as was MTOB, compared to HIPP. With regard to access in the assay, a single CtBP molecule most likely first contacts an NADH molecule, forming the ordered reaction necessary to dimerize. Then, of molecules that can fit within the substrate-binding domain, MTOB was the most abundant (up to roughly 50-fold excess under some conditions). HIPP would need to compete to find access to CtBP, and if NAD⁺ release was the rate limiting step, it would have the greatest opportunity to interact at that point. It is difficult to conjecture the state of CtBP molecules within cell systems, but we can perform some tests that will help us to understand inhibitor interaction both with enzymatic and cell based assays. Also, we can think of processes that would allow for us to promote complex disassembly. While the Discussion and Future Directions section of **Chapter 3** examines different therapeutic strategies, one thought is worth mentioning here. It is proposed that if

inhibitor binding does function to stabilize CtBP transcriptional complexes, a potential solution could involve flushing the system or organism with MTOB, followed by treatment with a CtBP-specific inhibitor. In this way, an increase in MTOB or other substrate may push the complexes for disassembly, giving HIPP access to sequester CtBP monomers. Appreciable work would be necessary to optimize the timing of such a regimen, so that inhibitor would be added after loss of NAD⁺ in the enzymatic reaction, but before reassembly of other complexes.

We were able to develop a hypothesis stating that for enzymatic function and dimerization, CtBP must undergo an ordered addition reaction wherein NADH first binds, to allow domain closure. MTOB can bind regardless of the presence or absence of NADH, but premature binding impedes NADH binding and domain closure. HIPP was simulated to inhibit as part of an abortive ternary complex. In order to test the branches of our hypothesis, an ideal strategy would involve running similar enzymatic reactions as those described in the results, but with pre-steady state, or burst, kinetics. Pre-steady state interactions measure the rate of a reaction within the first couple of seconds. The purpose of pre-steady state kinetics is to help understand early processes, such as the order of addition of components in a reaction or a redox reaction, with a single turnover.⁹¹ To this point, we have not been able to do pre-steady state kinetics, largely because we have lacked the instrumentation to do so. In order to capture order of addition, it is important to monitor a reaction before it can start moving to product. This requires that one both rapidly mix the reaction and nearly simultaneously read the results. With manual addition and mixing, along with our spectrophotometer, we cannot read a reaction in under 15 seconds, which is far too late for measuring the pre-steady state phase of the enzymatic reaction. With proper instrumentation we could use either NADH absorbance ($A=340\text{nm}$) or fluorescence (emission at 445nm) to study order of addition. By combining roughly equimolar

MTOB to enzyme, followed by the immediate addition of NADH, we should be able to see if MTOB inhibits NADH interaction with the protein. By then completing the experiment in reverse order of addition, it would be plausible to compare the experiments to determine whether CtBP requires an ordered reaction for proper NADH binding. Similar experiments can be carried out in the presence of HIPP.

With current resources available to the Grossman lab and colleagues, a number of experiments can be performed. First, by using crystal structures of MTOB or HIPP bound to CtBP without the cofactor NADH, we can determine if MTOB-binding by itself is capable of permitting a conformational change that closes or clamps CtBP. If it does not, our hypothesis that NADH is necessary for protein clamping gains more support. Also, while pilot experiments were not successful (data not shown), it should be possible to perform pre-incubation experiments with our enzymatic NADH disappearance assay that can give us answers similar to that of what is possible with pre-steady state kinetics. Incubating CtBP with equimolar amounts of MTOB and subsequent addition of NADH, in slight excess or equimolar concentrations as CtBP and MTOB will be compared to experiments that have either concurrent addition of NADH and MTOB, or NADH incubated first. Single absorbance measurements would be taken, instead of attempting to calculate initial velocity of the reaction. An alternative approach would consist of adding half the molar equivalent of MTOB or HIPP as CtBP2 protein and then perform order of addition experiments with an excess of NADH. In this way, one can ascertain similar types of data, but as a function of normal enzymatic function.

Other techniques are being pursued to learn more about the effect of inhibitors on oligomerization and transcriptional function. Early analytical ultracentrifugation experiments with purified protein have been performed in the presence of HIPP (data not shown). The

reference sample sedimentation, demonstrates that CtBP2 alone exists almost entirely as a dimer. With the addition of HIPP, we see the formation of a second peak showing monomer. This data is encouraging in that it suggests that HIPP can dissociate CtBP dimers. Experiments have yet to be performed to determine the sedimentation profile of CtBP and NADH in the presence of MTOB, to give a reference for the expected amount of dimer and monomer under normal conditions. Additionally, early work has been performed to set up co-immunoprecipitation (Co-IP) experiments involving two differently tagged CtBP2 vectors. With this assay we will be able to study whether addition of HIPP or other drugs disrupts interaction of two monomers in a cell based system. As will be seen in Chapter 3, luciferase experiments have been performed to look at whether CtBP-specific function has been alleviated in the presence of inhibitors. Finally, the Grossman lab is working to optimize chromatin immunoprecipitation (ChIP) assays for CtBP targets so that we can directly assess whether CtBP-scaffolded transcriptional regulatory complexes are being dissociated from their promoter regions in the presence of inhibitors. From preliminary ChIP experiments performed by Dr. Seema Paliwal, formerly of the Grossman lab, we have shown that HIPP treatment displaces CtBP from the pro-apoptotic BH3-only gene, Bik (**Fig. 2_6**). We endeavor to similarly validate the next generation of inhibitors that are discussed in Chapter 3.

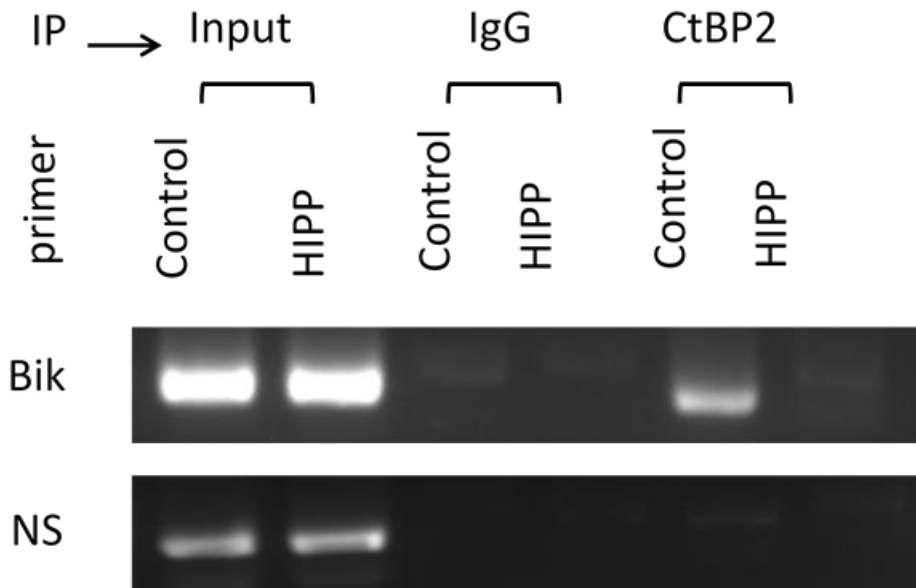


Figure 2_6. 2-Hydroxyimino-3-phenyl-propionic acid (HIPP) displaces CtBP2 from the Bik promoter. HCT116^{-/-} cells were treated for 72 hours with 1 mM HIPP or DMSO (control), and CtBP2 association with the Bik promoter assessed by ChIP using control IgG or anti-CtBP 2 antibodies and formaldehyde crosslinked chromatin. DNA retained in the ChIPs was analyzed by PCR using either Bik promoter primers that amplify a fragment containing BKLF sites (Bik) or a fragment of DNA located 10 kb upstream of the Bik transcription start site (NS).

Chapter 3: Manipulation of the HIPP backbone for future drug discovery

Rationale:

In the 2010 study demonstrating MTOB as a proof of principle CtBP-inhibitor, the Grossman lab was able to characterize the effect of MTOB cytotoxicity through cell based assays and mouse experiments.⁷⁰ For example, in HCT116 p53 null human colorectal cancer cell lines, MTOB was capable of displacing CtBP from the Bik promoter to induce Bik expression and apoptosis when MTOB concentrations were greater than 4mM. This same cytotoxic result was observed in sister HCT116 cells with functional p53. Knockdown of Bik, followed by treatment with 4mM MTOB almost entirely rescued cells from death, indicating that CtBP-mediated Bik de-repression was the primary mechanism of cytotoxicity in this system. Similarly, the lab group of Dr. Kevin Gardner has researched MTOB usage as a therapy in breast cancer cell lines.³⁰ Looking at a panel of 30 genes that were found to be CtBP-regulated in MCF-7 and MDA-MB-231 breast cancer cells, they saw that 10mM MTOB de-repressed 40 and 46% of genes, respectively with 70% concordance of the specific genes involved. In other words 70% of the genes that were de-repressed were shared between cell lines. By ChIP analysis, they showed that MTOB treatment displaced CtBP from 67% of its binding sites to promoters, which explained >70% of the gene expression changes in MCF-7 cell lines. In MDA-MB-231 cell lines, there was a smaller difference; MTOB caused 30% loss of CtBP promoter occupancy with a 50% concordance to the de-repressed gene function. This difference was attributed to lower CtBP levels seen in MDA-MB-231 versus MCF-7 cells.

Encouraged by our findings with regard to the discovery of HIPP, we examined HIPP induced cytotoxicity compared to that of MTOB. The results of the previous chapter gave insight into the relevance of rational design for discovering more potent inhibitors, and we have

proven that we can indeed identify a better class of inhibitors with our enzymatic assay. But the previous experiments did not demonstrate any cytotoxic effects or observations on phenotypic differences within cancer systems. Going forward in our experimentation, special emphasis was made to find drugs that were strong inhibitors as indicated not only by enzymatic assay, but also by demonstrating cytotoxicity to cells. We worked to create an even better series of drugs by deconstructing the HIPP structure and by substituting additional moieties. Two compounds, containing chlorine substitutions on the third and fourth carbon, respectively, on the phenyl ring of HIPP were shown to be significantly better enzymatic inhibitors and more cytotoxic agents than HIPP. We further performed luciferase assays to show a correlation with this cytotoxicity and relief of repression on the promoter of the CtBP-targeted pro-apoptotic gene Bik. In parallel, we sought to find treatment regimens that could be used in combination with CtBP-inhibitors to enhance cell death.

Materials and Methods:

Clonogenic Assays:

100,000 MDA-MB-231 breast cancer cells were plated per well and incubated overnight. Plates were treated with differing concentrations of drug or DMSO. Final DMSO concentration in each well was 0.5%. After 72 hours of drug treatment, media was aspirated, cells were rinsed in PBS, and fresh media was added. Cells were incubated a further 96 hours, and then plates were aspirated, rinsed in cold PBS, and cells were fixed in ice-cold methanol. Cells were then stained with a 0.05% crystal violet solution in PBS, rinsed and allowed to dry. n=3 independent replicates were performed.

Enzymatic Assays for CtBP Inhibitor Determination:

Assays were performed similarly to NADH disappearance assays in **Chapter 2**. Briefly, purified CtBP2 in 50% glycerol was added to 150 μM NADH, 48 μM MTOB and various concentrations of inhibitor in buffer containing 25 mM HEPES, pH 7.1, 25 mM potassium chloride, and 1 mM DTT. The final concentration of CtBP2 was 40 $\mu\text{g}/\text{mL}$ (986 nM) per reaction. Inhibitors dissolved in DMSO constituted 1% of the total volume. For compounds **4–13**, inhibitor concentrations ranging from 300 μM to 10 μM and a no-drug (DMSO) control were tested. For **9** and analogues **14a–m**, inhibitor concentrations of 5 μM , 2.5 μM , 1 μM , 500 nM, 100 nM, 50 nM, 10 nM, and 0 nM (DMSO control) were used. Three triplicate runs were performed for each inhibitor.

Reaction components were added to 96-well UV-Star Microplates (Greiner Bio-One) and upon addition of CtBP, reactions were mixed vigorously and immediately read by a Synergy H1 microplate reader (BioTek). Absorbance was recorded at $A = 340$ nm every 30 s for 15 min at 25 °C to measure CtBP2 dehydrogenase function (NADH, but not NAD^+ absorbs light at 340 nm). Change in absorbance was plotted after 15 min and IC_{50} concentrations were determined using Prism (Graphpad, Version 5.04).

Bik Luciferase:

HCT-116 $\text{p53}^{-/-}$ colorectal cancer cell (800 cells per well) were plated in a 96-well plate. After 24 hours, cells were transfected with pCDNA3-V5-CtBP2, the transcription factor pCDNA3-V5-KLF8, the firefly luciferase vector pGL3-Bik (promoter region -1710 to +203), and control luciferase plasmid expressing Renilla luciferase, pRL-TK, using Lipofectamine 2000 (Thermo Fisher). Following a further 24-hour incubation, vehicle (DMSO) or inhibitors (**9**, **14g**, **14h**, **14f**, **14k**, and **1**) were added to their final concentrations (1, 2, or 4 mM) and the expression

of luciferase reporter genes was determined using the Dual Luciferase assay (Promega). For analysis, firefly expression was normalized to Renilla control, and values were reported as a proportion of the luciferase expression of the vehicle (DMSO) control. Statistical analyses were determined by One-way ANOVA using a Tukey's post-test using a statistical package by Navenda Vasada (statistica.mooco.com), comparing 2mM, 4mM and no drug concentrations. Data points display the average of three independent experiments, and error bars represent standard deviation.

MTT Cell Viability Assays:

To measure cell viability with new inhibitors, HCT-116 p53^{-/-} colorectal cancer cell (~1,000 cells) in 100 μ L media were plated in a 96 well plate. After a 24 hour incubation, inhibitors in DMSO were further diluted in NaHCO₃ (0.8% diluted DMSO final volume/well) and added to plates at 4, 2, 1, and 0.5mM concentrations. 72 hours after addition of inhibitor, 20 μ L of MTT solution (Alfa Aesar) was added to each well and cells were incubated a further 4 hours. Media was then aspirated and the MTT metabolic product formazan was resuspended in 200 μ L DMSO. Optical density was measured at 560 nm (subtracting background at 670 nm) using a microplate reader and IC₅₀ concentrations were determined using Prism (Graphpad, Version 5.04).

For combination experiments with 4-Cl HIPP/3-Cl HIPP and MTOB, assays were performed as above, with concentrations as specified in the figure legend. For the various cell lines used to determine HIPP IC₅₀, experiments were performed similarly, varying initial cell counts according to growth rate and size to assure that no treatment cells were not confluent 72 hours post drug treatment. These assays contained contributions from various members of the Grossman lab.

Results:

HIPP cytotoxicity

Since HIPP was shown to be a significant inhibitor of CtBP, we endeavored to determine whether it caused cytotoxicity with equal potency to MTOB. HIPP was evaluated at various concentrations to generate cytotoxic EC50 values for several cell lines (**Fig. 3_1A**).

Interestingly, cytotoxicity coordinated with the cell types involved. Ovarian and colon carcinoma cell lines were most susceptible to HIPP treatment, whereas breast cancer and sarcomas were most resistant to the drug. Of note, the EC50 of HIPP in HCT116 p53^{-/-} cells was similar to that seen with MTOB⁷⁰. Further investigation by clonogenic formation assay shows that MTOB exhibited a slightly more potent effect long term (**Fig. 3_1,B-C**). Anecdotally it has been observed that MTOB does not show demonstrable levels of cell death until later points in time, but it appears to be more cytotoxic in long-term assays.

Discovery of more potent inhibitors of HIPP through analogue design

While HIPP was a more powerful inhibitor than MTOB, it did not demonstrate a more potent effect in cell-based assays. We therefore worked to design a better class of inhibitor that was also significantly more cytotoxic to cells through CtBP-specific avenues. In order to do so, different ketone-isosteres were produced using the backbone structure of phenylpyruvate (**Fig. 3_2A**). This was done to determine whether the imine of HIPP produced the most specificity for enzymatic interaction, and the moiety was replaced with an α -thioketone, acrylic acid, malonic acid, amide, and hydrazone (**Fig. 3_2A**, compounds 4-8, in order). Interestingly, none of these compounds, with the exception of HIPP, and to a weaker degree phenylpyruvate, showed enzymatic inhibition through an NADH disappearance reaction (**Fig. 3_2C**).

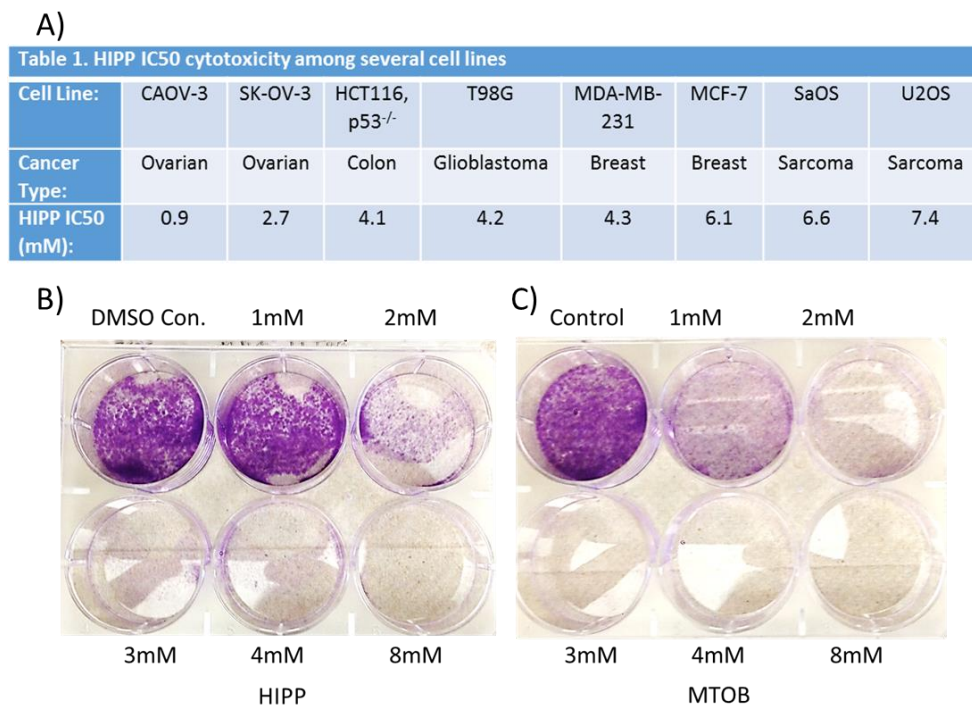


Figure 3_1 HIPP cytotoxicity in multiple cancer cell lines A) Healthy cell viability was assessed at several drug concentrations and analyzed after 72 hours by MTT assay. IC50 was measured using nonlinear regression analysis by Graphpad Prism. Work was contributed by several members of the Grossman lab. Clonogenic assay involved addition of drug for 72 hours, followed by 96 hours no treatment. Representative results are shown for HIPP B) and MTOB C).

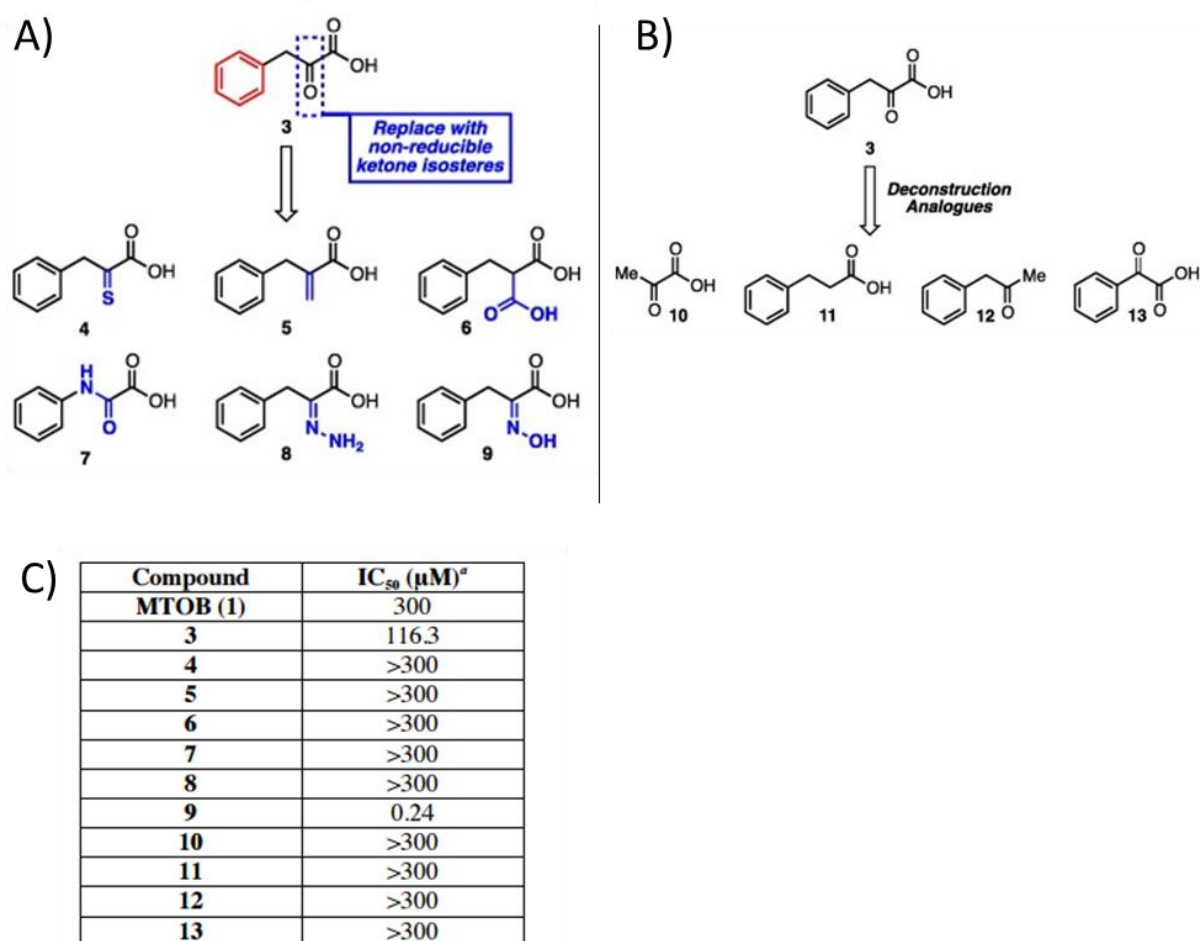


Figure 3_2. Replacement of the HIPP imine and deconstruction analogues of phenylpyruvate to identify key components contributing to enzymatic inhibition.

Compounds were either synthesized by Dr. Sudha Korwar and Dr. Keith Ellis or purchased for testing. **A)** The ketone of phenylpyruvate was substituted with other moieties to look for isosteres that could increase inhibition potential. **B)** Phenylpyruvate was further deconstructed to identify which components were imperative for inhibition. **C)** Enzymatic IC₅₀s were calculated with an upper limit of 300μM drug. All assays were performed in duplicate, with triplicate measures.

Using a similar approach, deconstruction analogues of phenylpyruvate were also designed. These included removing the phenyl ring (pyruvate), the ketone (hydrocinnamic acid), the carboxylic acid (1-phenylpropan-2-one), and shortening the carbon chain by removing the methylene spacer between the phenyl ring and the α -keto acid (**Fig. 3_2B** compounds 10-13, in order). Similar to the ketone replacement compounds, deconstruction of phenylpyruvate eliminated inhibition activity. Therefore, of the compounds that we tested, HIPP was the most potent inhibitor, and each moiety studied, including the phenyl ring and imine, appear to be important for optimized CtBP inhibition.

Knowing that HIPP provides the best basic structure for inhibitor design, a series of compounds was designed, creating substitutions along the aromatic ring. These compounds were validated as potential inhibitors using docking simulations with the CtBP-HIPP crystal structure (performed by Dr. Glen Kellogg), and 13 of the best, based off HINT scoring function, an algorithm which correlates with free energy of binding,⁹² were synthesized. These various substitutions are found in **Figure 3_3**. The number preceding each substitution indicates to which carbon of the phenyl ring it was attached.

We evaluated these compounds using NADH disappearance assays to test for enzymatic inhibition and MTT assays to assess for differences in cell viability in the presence of drugs. A summary of these results are found in **Table 1** and examples showing raw data values with some of our best inhibitors are found in **Figure 3_4,A-C**. Several general characteristics can be observed from these new compounds. Of note, substitutions on carbon C-2 are poor inhibitors in enzymatic assays, with the best of these, 2-Cl HIPP, inhibiting at a concentration of 7.65 μ M. By comparison the best inhibitors, 3-Cl HIPP and 4-Cl HIPP have enzymatic IC₅₀'s of 0.17 and 0.18 μ M respectively. One derivative, 2-OMe HIPP, was found to be incapable of inhibiting

CtBP enzymatic function, with an IC₅₀ beyond the upper limits of the assay. However, the compound was comparatively very cytotoxic in HCT116 p53^{-/-} cells, with a cellular EC₅₀ of 1.24 mM. 3-OH and 4-OH HIPP both had cellular EC₅₀'s extending beyond the upper limit of ranges tested (10mM). Together, we chose those compounds that were both more cytotoxic and better inhibitors (**Fig. 3_4D**) as potential lead compounds for further analysis. Of those compounds evaluated, 3-Cl HIPP and 4-Cl HIPP were the two compounds that met this criteria (see **Table 1**), with cellular EC₅₀ values of 0.85 and 1.74mM.

We sought to understand the differences between cell viability and enzymatic IC₅₀. The cLogP values (**Table 1**), which measure the difference in solubility between octanol and water, was found to be less than 3 for each compound. This value indicates they are likely cell permeable, and meet the threshold for suggested lead compounds in drug design.⁹³ Additionally, Dr. Sudha Korwar performed a high performance liquid chromatography (HPLC) experiment for HIPP, 3-Cl HIPP, and 4-Cl HIPP for a period of 3 days to monitor degradation of the compounds. After 72 hours no degradation was observed, suggesting that the compounds were not hydrolyzed in aqueous conditions.

While the two new lead compounds, 3-Cl and 4-Cl HIPP demonstrate a discrepancy between viability and inhibition, they still show 4-fold higher cytotoxicity than either HIPP or MTOB. To assess whether this decrease in cell viability may be due to CtBP inhibition, we performed Bik luciferase experiments in HCT116, p53^{-/-} cell lines. Bik is a gene that is normally repressed by CtBP and encodes a BH3-only pro-apoptotic protein that, when expressed, induces apoptosis through the caspase-9 mitochondrial system.⁵⁶ CtBP downregulation leads to Bik expression. The subsequent Bik-induced cell death is a significant source of CtBP-mediated apoptosis.⁵⁷ In this experiment, the Bik promoter was attached to a firefly luciferase gene. We

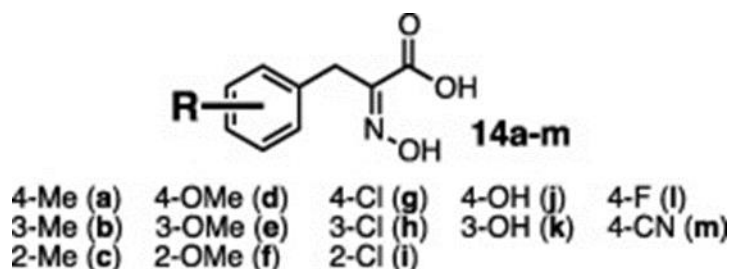


Figure 3_3. Analogues of HIPP designed to explore structure-activity relationship.

Substituents were placed within the phenyl ring of HIPP (R-). The numbers preceding each substitution specify onto which carbon of the phenyl ring it is placed.

Compound	Substituent	HINT score	CtBP IC ₅₀ ^a (μM)	Cellular IC ₅₀ ^a (mM)	C Log P
9	H	963	0.24 (0.21, 0.27)	4.12 (2.96, 5.73)	1.36
14a	4-Me	946	0.32 (0.29, 0.37)	3.28 (2.51, 4.28)	1.86
14b	3-Me	756	0.48 (0.43, 0.54)	3.26 (2.71, 3.93)	1.86
14c	2-Me	1078	8.73 (6.19, 12.29)	0.23 (0.16, 0.35)	1.81
14d	4-OMe	925	2.16 (1.19, 3.90)	1.93 (1.65, 2.25)	1.28
14e	3-OMe	919	0.88 (0.81, 0.97)	5.60 (3.56, 8.84)	1.28
14f	2-OMe	858	>100	1.24 (1.02, 1.51)	1.28
14g	4-Cl	861	0.18 (0.16, 0.20)	1.74 (1.47, 2.06)	2.07
14h	3-Cl	844	0.17 (0.15, 0.19)	0.85 (0.76, 0.96)	2.07
14i	2-Cl	974	7.65 (5.93, 9.86)	2.37 (1.83, 3.08)	2.07
14j	4-OH	767	7.34 (5.26, 10.25)	>10	0.69
14k	3-OH	1003	0.72 (0.67, 0.78)	>10	0.69
14l	4-F	n.d. ^b	0.30 (0.27, 0.33)	3.97 (3.52, 4.49)	1.50
14m	4-CN	574	0.90 (0.82, 0.98)	1.10 (0.81, 1.49)	0.79
MTOB (1)	—	n.d. ^b	n.d. ^b	4.0 ^c	-0.34

^a Data represents the averages with 95% confidence intervals shown in parentheses of three independent experiments.

^b n.d. = not determined.

Table 1. Inhibition of HIPP analogues by enzymatic reaction and cell viability in HCT-116 p53-/- colon cancer cells, along with a measure of hydrophilicity, cLogP. The cellular IC₅₀ for MTOB was determined in reference 68.

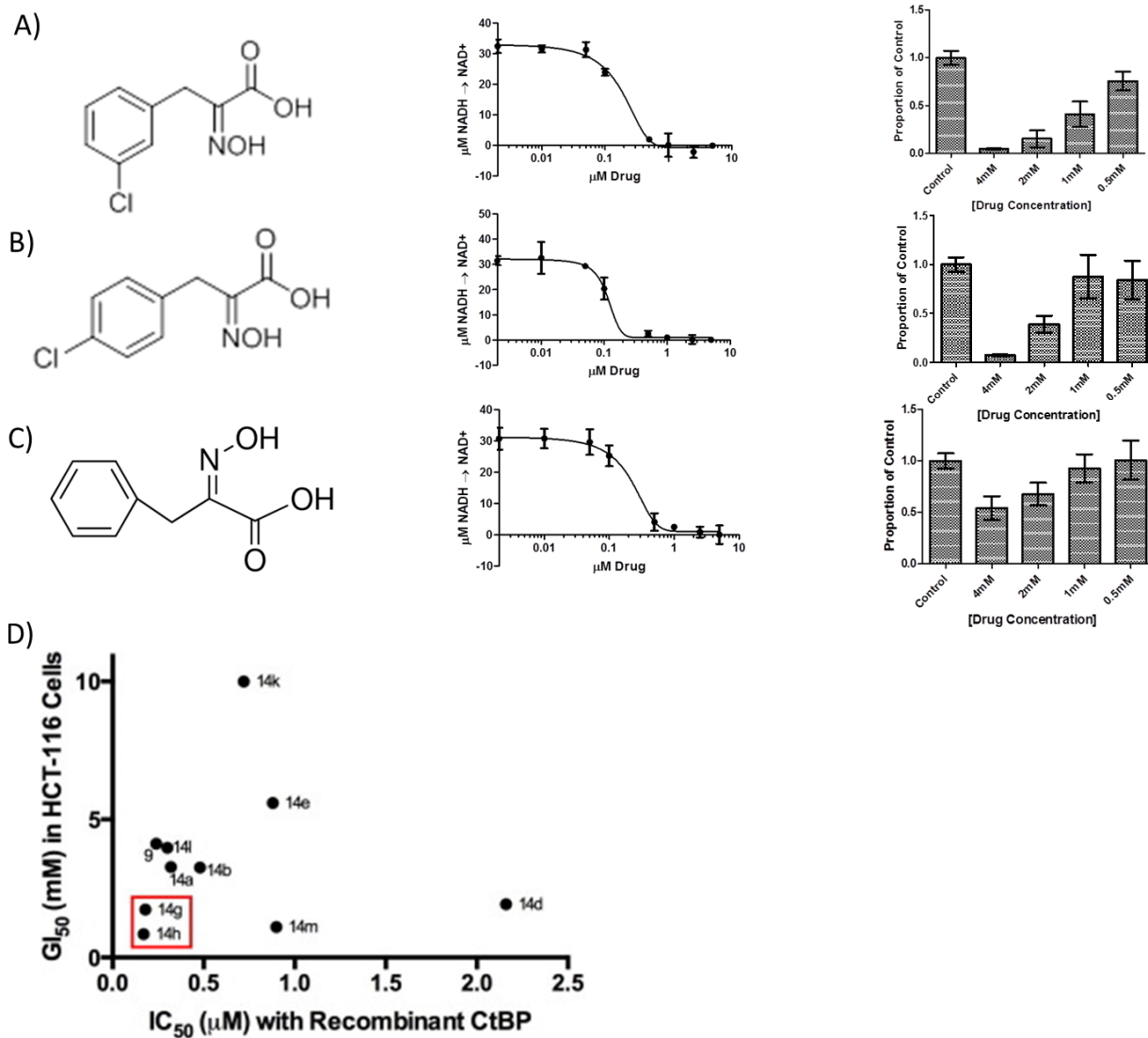


Figure 3_4. Generation of enzymatic IC₅₀ and cell viability EC₅₀ concentrations for Phenyl-ring substitution analogues of HIPP. Enzymatic IC₅₀ curves (middle panel) showing the log range of drugs used for **A)** 3-Cl HIPP, **B)** 4-Cl HIPP, and **C)** HIPP. Also shown are the averages used to generate an EC₅₀ for cell viability. **D)** A plot showing the enzymatic IC₅₀ values on the X-axis vs. the cellular EC₅₀ on the Y-axis. Numbers in D, represent labels as shown in **Table 1**. 3-Cl HIPP (14h) and 4-Cl HIPP (14g) are outlined in red. For all data, n=3 independent experiments, in triplicate.

expect that CtBP-specific inhibitors will derepress the Bik promoter, and better inhibitor function will be associated with higher Bik-luciferase levels. In order to capture Bik expression without inducing cell death, we performed experiments 24 hours after drug addition, using a range of concentrations that incorporate the prescribed EC50 cytotoxicity values generated from a 72 hour time point.

The Bik luciferase experiment was performed with 0, 0.1, 0.2, 0.5, 1, 2, and 4mM drug concentrations. 3-OH HIPP was used as a negative control, as it was shown to inhibit CtBP in enzymatic assays, but was not cytotoxic at the levels tested in this assay (**Fig. 3_5A**). 2-OMe HIPP was also used as a control that exhibits cytotoxicity, but is not an inhibitor of CtBP. 2-OMe HIPP, 3-Cl HIPP, and 4-Cl HIPP luciferase activity could not be calculated at 4mM concentrations due to significant cell death. After 24 hours with 2mM drug concentration, both 3-Cl HIPP and 4-Cl HIPP expressed significantly higher Bik concentrations than any other drugs at either 2mM or 4mM concentration ($p < 0.01$, **Fig. 3_5B**). The fold change over the no-drug control for Bik luciferase was as follows: 3-Cl HIPP showed 1.41-fold increase at 1mM concentration and 2.98-fold increase at 2mM; 4-Cl HIPP had a 1.32-fold increase at 1mM and a 2.9-fold increase at 2mM. Comparatively, HIPP had a maximum fold induction of 1.8 fold at 4mM concentrations. MTOB showed only marginal increases in Bik luciferase expression over the no-drug control.

It is important to note the results of our controls. 2-OMe HIPP, at the highest dose tested that did not significantly kill cells, showed only a modest 1.45-fold induction of Bik promoter activity. This was not found to be statistically significant versus the no-drug control. 3-OH HIPP showed an increase in luciferase expression at 4mM concentrations (1.6-fold); however, it was likewise not statistically significant.

Together, this luciferase data fits with our hypothesis and correlates strongly with the enzymatic inhibition profiles that were generated. Neither 3-OH HIPP nor 2-OMe HIPP showed significant luciferase expression, even though 2-OMe HIPP induced cell death at the highest levels measured. Due to the lack of enzymatic CtBP inhibition or Bik luciferase expression, it is hypothesized that 2-OMe HIPP induces cell cytotoxicity through a CtBP-independent mechanism, that has yet to be investigated. 3-Cl HIPP and 4-Cl HIPP had the highest luciferase expression, and the only expression that was statistically significant. This result suggests that this increased cytotoxicity is due, at least in part, to CtBP inhibition. While these compounds do not reach cytotoxic potentials that can be considered therapeutically efficacious, the ability to further improve upon HIPP is encouraging and suggests that we have a viable rationale for future drug design.

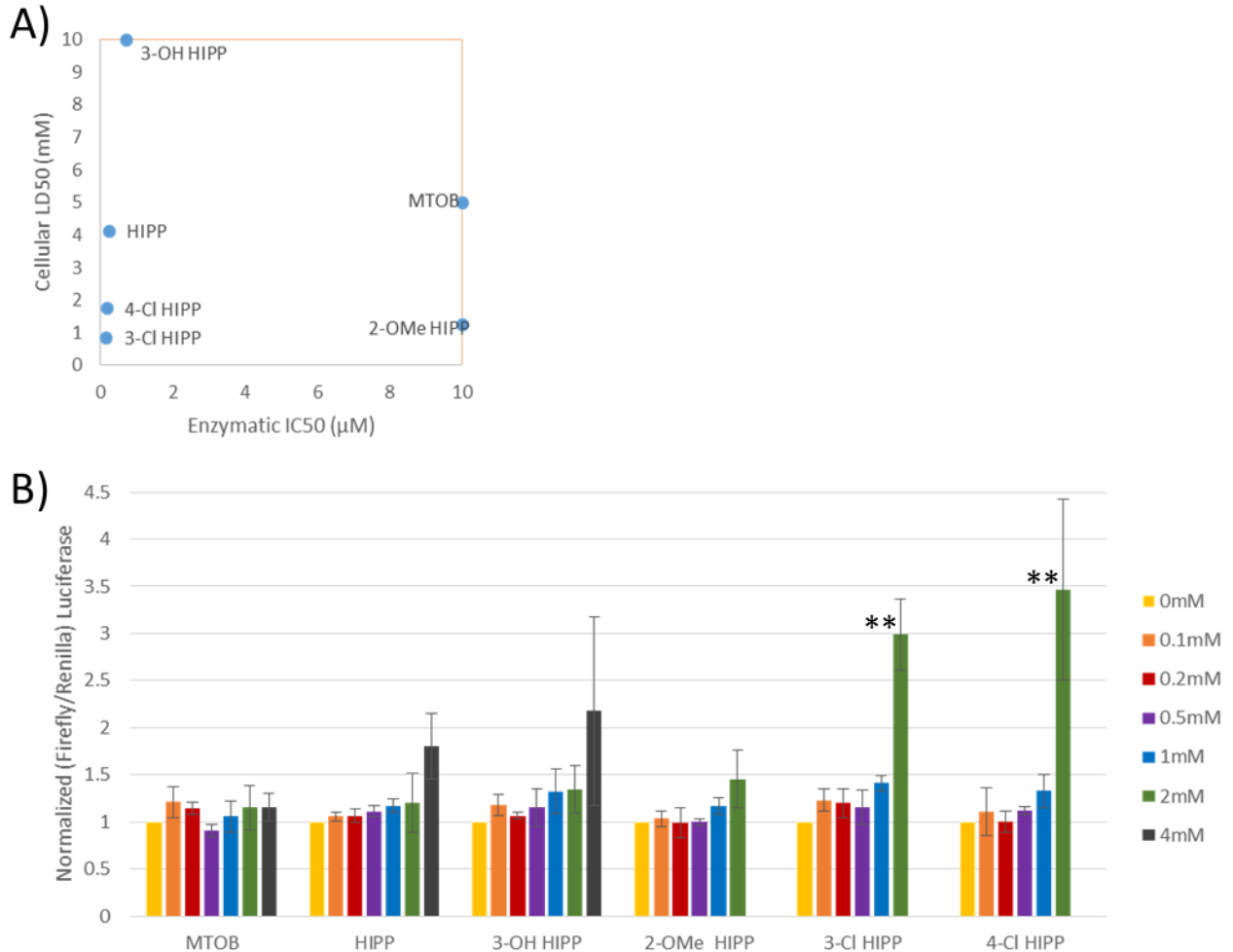


Figure 3_5. Treatment with drugs shows variable expression of the CtBP-repressed Bik promoter in luciferase assays. **A)** An adaptation from **Fig. 13D** showing the molecules carried forward to luciferase expression. Points intersecting the orange axis represent IC₅₀ outside the range of the assay. 3-OH HIPP is a CtBP specific, but non-cytotoxic inhibitor. 2-OMe HIPP is cytotoxic, but not CtBP specific. **B)** The pro-apoptotic Bik-promoter was fused to firefly luciferase and measured following 24 hours of drug treatment in HCT116, p53^{-/-} transfected cells. Values were normalized to a renilla luciferase internal control and a 0.5% DMSO, no drug control, was set to 1. 2-OMe HIPP, 3-Cl HIPP, and 4-Cl HIPP were cytotoxic at 4mM concentration and luciferase expression could not be determined. ** = $p < 0.01$ vs all other 2mM and 4mM drug concentrations, by Tukey's post-test following One-Way ANOVA. No other statistical significance was found between other groups at 2mM, 4mM concentrations or versus the no-drug control.

Combination experiments for more potent therapeutic strategies:

As new drugs were developed, we made efforts to test compounds in combination with other therapeutics. One of the first experiments performed was with MTOB in conjunction with chemotherapeutic reagents. This extended from earlier published experiments with MTOB and cisplatin in which cisplatin and MTOB combined to exhibit an additive effect.⁷⁰ Cisplatin functions by entering cells and creating reactive oxygen species.⁹⁴ It also intercalates with purine residues on DNA to induce DNA damage and halt cell cycle progression. Oxaliplatin is similar in structure, but is characterized by rapid displacement of an oxalate group that causes DNA damage.⁹⁵ It can create inter-strand crosslinks similar to cisplatin, but the major mechanism of action is believed to be caused by intra-strand reactions between guanines, or occasionally guanine-adenine interactions. Gemcitabine, on the other hand is considered an anti-metabolite. A di-phosphorylated form of the molecule limits a cell's deoxyribonucleotide (dNTP) pool by inhibiting a molecule, RRM1, that is necessary for nucleoside recycling. It further intercalates into elongating DNA, blocking base-excision repair and inducing downstream apoptosis.⁹⁶

Using Clone-A colon cancer cells, we performed similar experiments with oxaliplatin and gemcitabine as to those done with cisplatin, by varying MTOB concentration while keeping the concentration of chemotherapy constant (**Fig. 3_6**). We saw roughly 50% cell death with 4mM MTOB after 72 hours, while gemcitabine was effective in reducing cell viability to about 30% with 500nM drug. Oxaliplatin showed roughly 70% viability with a 2 μ M concentration. Combinations failed to show an additive or synergistic effect. In fact, addition of MTOB to gemcitabine increases the cell viability above that of gemcitabine treatment alone. Low levels of MTOB with oxaliplatin seem to function similarly. At the highest concentration of MTOB,

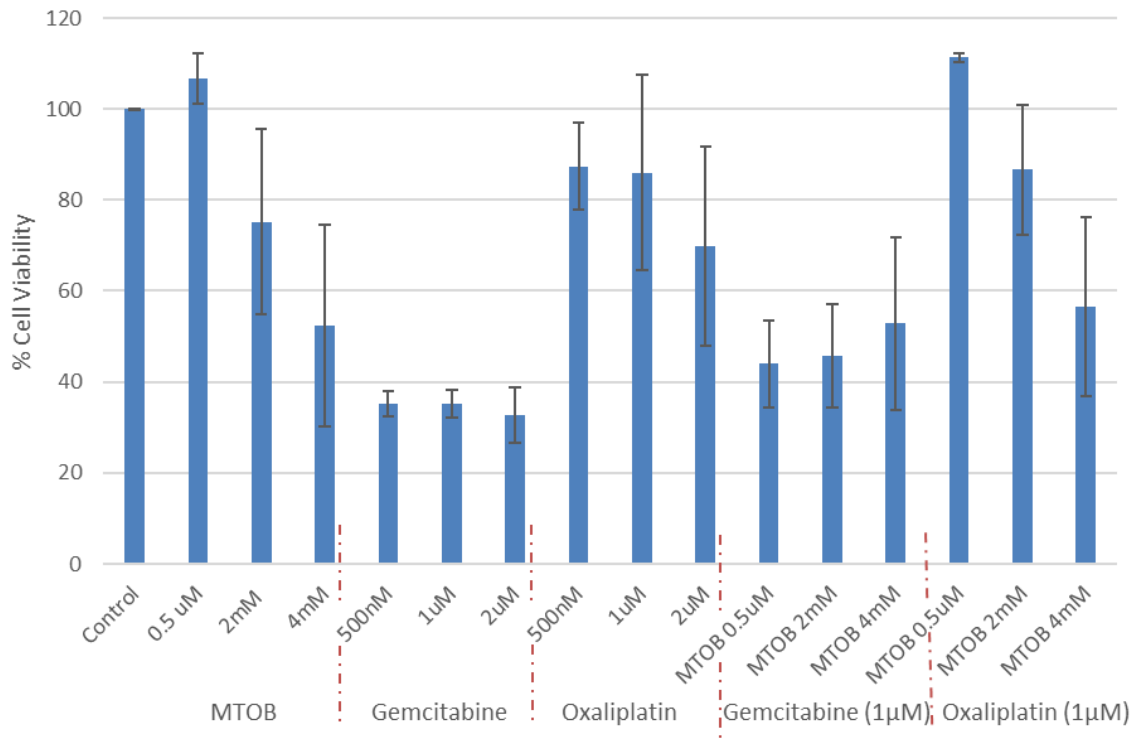


Figure 3_6. Combination therapies using chemotherapeutic agents with MTOB to measure cell viability. Using Clone-A cells, we performed MTS assay, in the presence of DMSO control, MTOB alone, gemcitabine or oxaliplatin alone, or MTOB and gemcitabine/oxaliplatin together 72 hours post inoculation. n=2 independent biological replicates with triplicate measures.

oxaliplatin did not show a greater collaborative effect than the amount of cell death seen alone with MTOB at 4mM drug concentration. These results differed from expectations, and may be attributed to a couple of factors. For one, the original cisplatin experiments used HCT116 colorectal cells, instead of Clone-A cells and so results cannot be compared directly. Also, cisplatin has been indicated to activate HIPK2, a kinase that can phosphorylate CtBP serine residue S428 of CtBP1.^{29,61,62} The phosphorylation event targets CtBP to proteasomal degradation. This may provide cisplatin an advantage over other chemotherapeutics by also indirectly targeting CtBP-mediated degradation. Regardless, under these conditions, MTOB does not complement gemcitabine or oxaliplatin as a treatment strategy in this cell type.

Despite the lack of synergy or additive effects between MTOB and chemotherapeutic agents in cell culture systems, Dr. Barur Rajeshkumar, a former postdoc of Dr. Grossman at the University of Massachusetts Medical School, performed preliminary mouse experiments with HIPP. He first determined the maximum tolerated dose to be 250mg/kg. Unlike MTOB, which is highly tolerated in mice in doses exceeding 750mg/kg, HIPP does exhibit lethality. Taking this understanding forward, he took two sets of *Nu/Nu* mice and injected them with either 3×10^6 Clone-A or HCT116, *p53*^{-/-} colon cancer cells. After a week, the mice were started on a regimen of IP injections with PBS, 250mg/kg gemcitabine, 50mg/kg oxaliplatin, 250mg/kg HIPP, or combinations of chemotherapy and HIPP (250mg/kg each of gemcitabine and HIPP, or 50mg/kg of oxaliplatin with 250mg/kg HIPP), once a week for a total of 8 weeks. After this time, mice were sacrificed and tumor volume was measured. In mice xenografted with Clone-A cells (**Fig. 3_7A**), HIPP and gemcitabine had a somewhat additive effect, with the combination of treatments forming significantly smaller tumors than PBS treated mice. Oxaliplatin and HIPP co-treated mice, by contrast, did not see any benefit in the relief of tumor

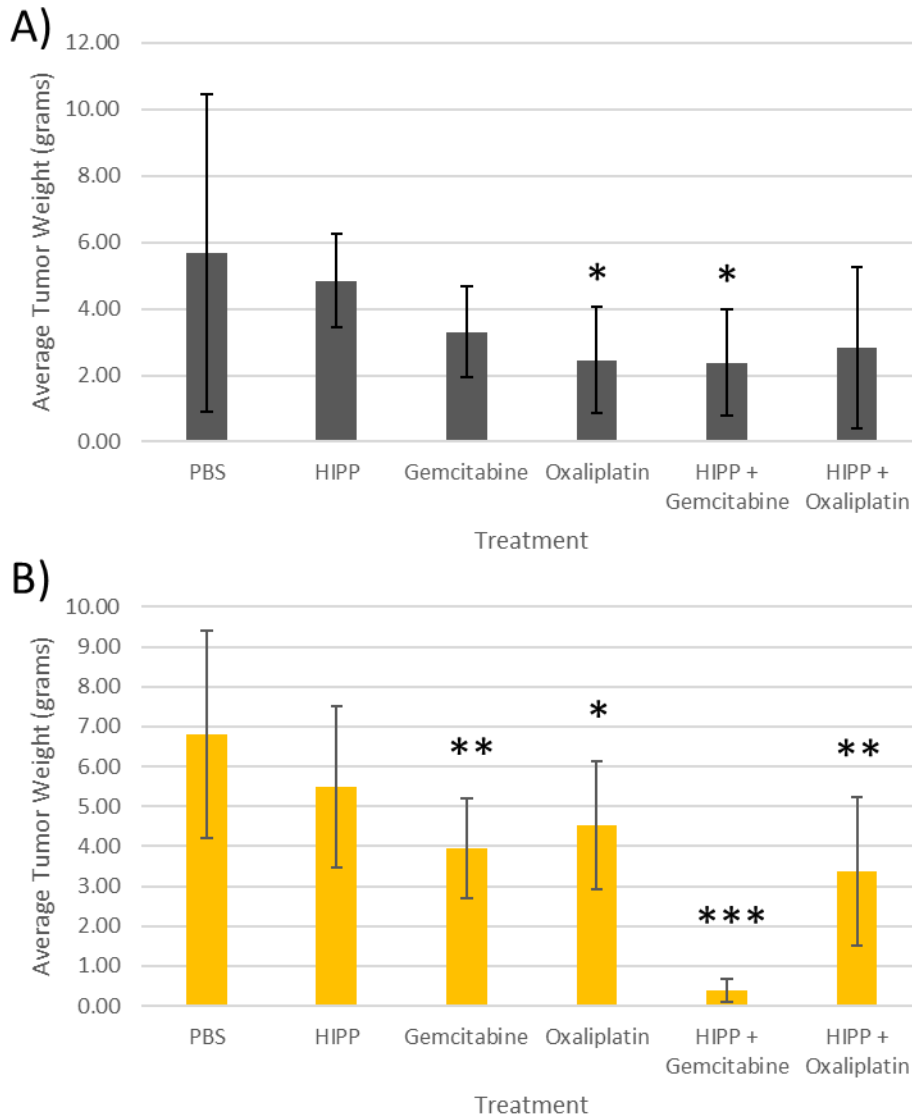


Figure 3_7. Combination therapies to reduce tumor burden in immunodeficient mice. Nu/Nu mice were inoculated with 3×10^6 Clone-A colon cancer cells **A)** or HCT116 human colorectal cancer cells **B)**. After 1 week, they were treated weekly by intraperitoneal injection (IP) with either 250mg/kg PBS, oxaliplatin, gemcitabine, HIPP, or combination of HIPP and chemotherapy. After 8 weeks, mice were sacrificed and tumor weight was measured. n=14 mice began treatment for each group. *=p<0.05 vs PBS control; **=p<0.01 vs PBS control; ***=p<0.01 vs all other treatments by Tukey's post-test.

burden versus oxaliplatin treatment alone. Mice xenografted with HCT116 cells illustrated a more substantial phenotype (**Fig. 3_7B**). Oxaliplatin and gemcitabine alone were capable of lowering tumor weight compared to a PBS control. Oxaliplatin in combination with HIPP showed a slight combined effect in decreasing tumor weight, but it was not significantly more than oxaliplatin alone. Gemcitabine and HIPP treatments, however, combined to show an apparently synergistic effect. Many mice did not have tumors that could be weighed by the end of the treatment and the average weight of tumors in this group was 0.38 grams. Gemcitabine treated mice had an average tumor weight of 3.95g and HIPP treated mice had an average of 5.49g. PBS treated mice carried tumor burdens weighing 6.80g. Subtracting the difference in weight between gemcitabine-only treated mice and HIPP-only treated mice, compared to PBS controls, we see a combined difference in tumor weight of 4.16g. The combination therapy showed a difference in weight of 6.42g versus PBS controls. This suggests that indeed, there is a synergistic effect seen with these two drugs combined. Heartened by these results, mouse studies are being performed using 4-Cl HIPP. The first studies will evaluate the maximum tolerated dose of 4-Cl HIPP before assessing its tumor anti-proliferative effect as a therapy, both alone, and in combination with chemotherapeutics and other drugs.

Another avenue for combination therapies could involve using two drugs that target the same basic pathway in the hopes of seeing a more complete ablation of that pathway's phenotype. CtBPs are known to incorporate a number of different transcriptional regulators to control gene expression. One group of proteins that CtBP recruits is histone deacetylases, or HDACs. As the name implies, HDACs remove acetyl groups from histones, which tightens DNA wrapping along the histone, making DNA much less accessible to transcriptional

machinery. Inhibitors have been developed to target aberrant de-acetylation. One inhibitor in particular, suberic bishydroxamate (SBHA) is an inhibitor that targets cancer systems, through mediating the upregulation of Bcl-2 family proapoptotic proteins, such as Bim, Bax, and Bak⁹⁷. CtBP has also been shown to repress Bax and other Bcl-only proapoptotic genes^{29,53}. We therefore hypothesized that inhibitors for both of these protein families would target a similar pathway. If so, we could account for insufficient repression of one set of molecules by repression of the other to create an additive effect. To test this idea, we performed an MTT cell viability assay, as before, in H1299 cells (**Fig. 3_8**). We saw the effect of MTOB or SBHA alone, or in tandem. The results fail to show a clear additive effect with concentrations of MTOB as high as 1mM and SBHA as high as 30 μ M. Mild levels of additive effects can be seen with lower concentrations of SBHA and 1mM MTOB, but the results are very subtle. One interpretation of this result is that one inhibitor is sufficient to target the Bcl-2 family proapoptotic gene pathway; the addition of another inhibitor is redundant, but does not enhance targeting. The result differs from expectations, but is nevertheless informative.

Lastly, we sought to incorporate our newest inhibitor leads, 3-Cl HIPP and 4-Cl HIPP in combination therapies. We specifically hypothesized that MTOB could be used in conjunction with these inhibitors to take advantage of the enzymatic function of CtBP. We postulated that at high levels, MTOB would be able to enforce a dehydrogenase reaction between itself and NADH, which would then cause release of MTHB and NAD⁺, allowing disassembly of CtBP complexes. Concurrently flushing the cells with our HIPP analogues would allow binding to the CtBP monomeric state with much higher affinity than MTOB alone before CtBP had the opportunity to reform complexes. As mentioned previously, HIPP has a ~1000-fold higher

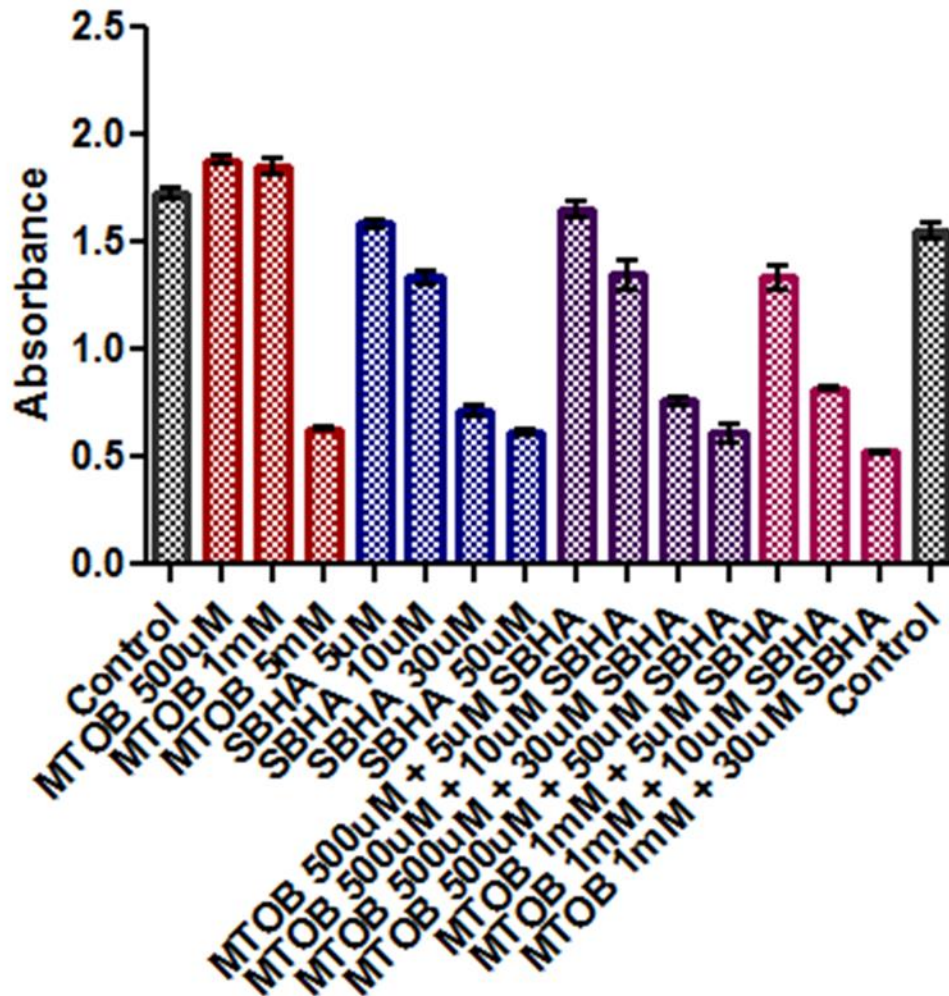


Figure 3_8. Combination therapies of MTOB with the HDAC inhibitor Suberohydroxamic Acid (SBHA). Cell viability was determined in H1299 lung cancer cell lines by drugging cells that had been plated overnight. After 72 hours drug treatment, viability was measured by MTS assay. Cells were treated with MTOB alone, SBHA, or in combination to test for additive or synergistic effects. A second control was included to illustrate that differences were not due to plate effects. Absorbance was measured at $A=490\text{nm}$ and is a measure of living cells.

affinity than MTOB (MTOB $K_d=2.96\text{mM}$, HIPP $K_d=1.30\mu\text{M}$), and although K_d had not been specifically measured for 3-Cl HIPP and 4-Cl HIPP, we assumed that they would have similar affinities based off their more inhibitive enzymatic IC_{50} values. Our assay alone cannot prove the hypothesis that MTOB and HIPP combination would induce monomeric CtBP isolation followed by monomeric sequestration, but significant data with MTOB and HIPP, would provide the impetus for further investigation.

The results of the screen are seen in **Figure 3_9A** for 4-Cl HIPP and **Figure 3_9B** for 3-Cl HIPP. Note that the y-axis measures living cell viability. Briefly, HCT116 cell lines were again propagated and plated for MTT assay. Both MTOB and HIPP derivative drugs were varied across a panel of concentrations and in different combinations, and the plates were incubated for 72 hours before being read by MTT assay. 3-Cl or 4-Cl HIPP concentrations were 0, 0.01, 0.05, 0.1, 0.5, and 1mM. MTOB concentrations were 0, 0.1, 0.5, 1, and 2mM. For the sake of clarity, only 0mM (orange bars) and 1mM (grey bars) MTOB concentrations are shown. For lower concentrations of MTOB no significant effect was seen. But with a concentration as low as 1mM MTOB, there was a significant difference seen with combination therapy with 50 μM 4-Cl HIPP compared to 4-Cl HIPP alone (**Fig. 3_9A**, $p=0.03$ by one-sided paired t-test). Similarly, 1 μM 4-Cl HIPP with 1mM MTOB trended toward significance ($p=0.069$). It is likely that further investigation will yield more power to understand the significance of this observation, as the data only represents two independent replicates, each measured with triplicate wells. However, it should be noted that neither concentration alone is sufficient to cause cell death. In HCT116 cells, the IC_{50} for MTOB treatment is over 4mM. In the 0mM MTOB control for 50 μM 4-Cl HIPP shows no decrease in cell survival. Thus, we see significant cell

death using a roughly 4-fold lower concentration of MTOB and a 3-fold lower concentration of 4-Cl HIPP than what has been necessary to generate 50% cell cytotoxicity alone.

Interestingly, this theme is not observed for 3-Cl HIPP (**Fig. 3_9B**). More work is required to: first, assure the validity of this data through further replication; second, tease apart the conditions in which combination therapy with MTOB may be useful so that it can be translated to an *in vivo* system; and third, perform assays that will be informative as to whether this treatment regimen functions according to the hypothesized mechanism. There remains the possibility that this treatment may work by some hitherto unknown pathway. The discussion investigates potential assays that can interrogate these goals. Altogether this research has allowed us to branch substantially from looking at a single therapy regimen for drug treatment and continued drug discovery.

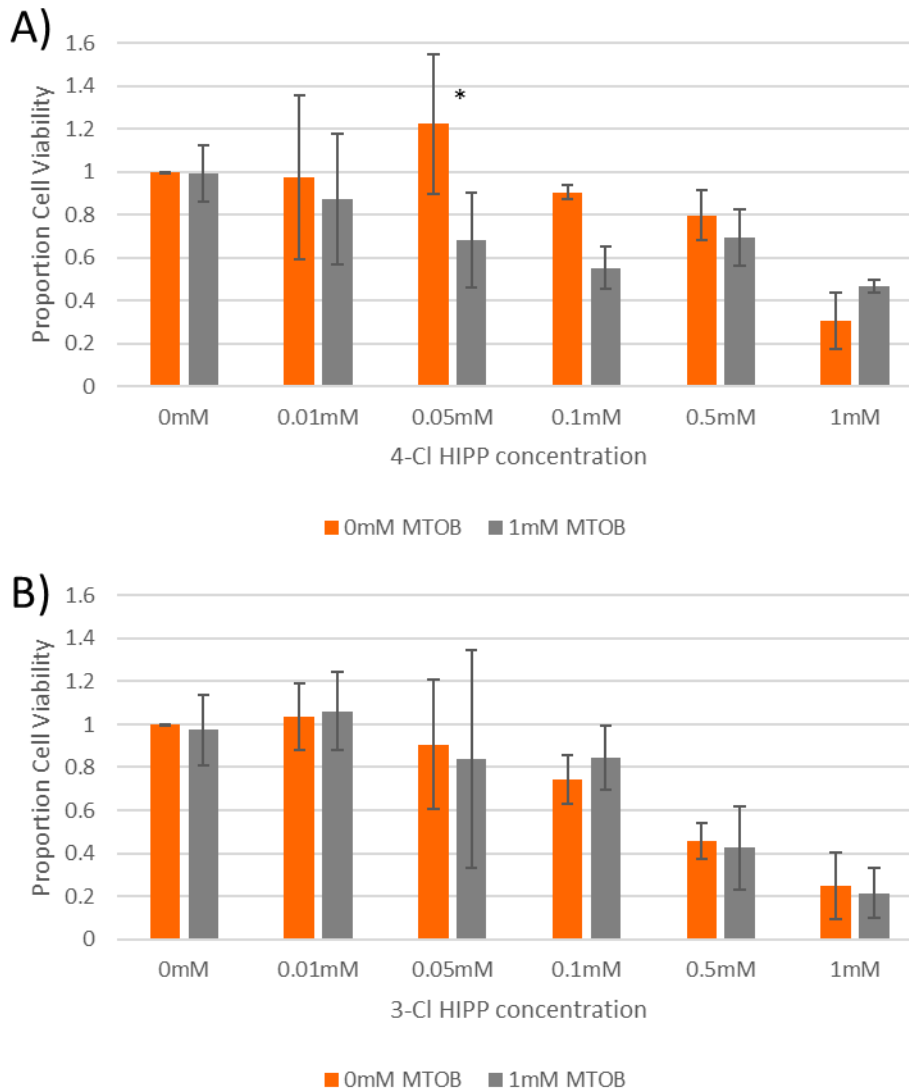


Figure 3_9. Combination therapy of 4-Cl and 3-Cl HIPP analogues with MTOB. MTT assay in HCT 116 p53^{-/-} cell lines, treated with varying concentrations of either 4-Cl HIPP **A)** or 3-Cl HIPP **B)** and MTOB for 72 hours. n=2 independent replicates of 3 wells each. *=p<0.05 by one-sided paired t-test.

Discussion and Future Directions:

Discovery and screening of more potent inhibitors based on the structure of HIPP

Through a detailed interrogation of the components of HIPP and phenylpyruvate, we discovered which regions are necessary for inhibition, and determined that the branch length, phenyl ring, carboxylic acid and imine moieties all contribute toward inhibitor function. Additionally, we were able to discover two drugs, 3-Cl HIPP and 4-Cl HIPP that are more enzymatically inhibitory (IC₅₀: 0.17 μ M and 0.18 μ M, respectively) than HIPP (0.24 μ M) and that, for the first time with a substrate-specific CtBP inhibitor, reached cytotoxic levels bordering on micromolar concentrations. (3-Cl HIPP: 0.85mM; 4-Cl HIPP: 1.74mM; HIPP: 4.12mM). Additionally, we were able to show this cytotoxicity likely occurs in part through de-repressing CtBP transcriptional function. By luciferase assay, it was observed that HIPP, 3-Cl HIPP, and 4-Cl HIPP each had the highest increase in Bik luciferase function, correlating with the combination of cytotoxicity and inhibition associated with each. 3-Cl HIPP and 4-Cl HIPP-mediated bik-luciferase expression was significantly elevated at a level nearly three-fold higher than controls. Importantly, our controls functioned as hypothesized. A cytotoxic, but not CtBP-specific, drug was able to kill cells at the highest concentration (4mM), but did so without increasing Bik luciferase expression. Second, a CtBP inhibitor that was not cytotoxic, 3-OH HIPP, did not show cytotoxicity and did not show significantly increased Bik levels. MTOB likewise did not induce apoptosis (typical MTOB-mediated apoptosis does not occur before 48hours), and as such, Bik luciferase levels were also low. This provides some weight to the merit of the luciferase assay, showing in this instance that nonspecific cell death does not raise Bik. Further, Bik expression is not elevated with CtBP-specific drug targets, except at

concentrations and under conditions that begin to promote apoptosis. This experiment raises new questions and new avenues for future research.

An example is seen with the newest batch of compounds. 3-OH HIPP was found to be a good inhibitor of CtBP function but was not found to exhibit significant cytotoxicity. This finding is interesting and it begs the question as to what is occurring during drug treatment. Looking at the cLogP value shown in Table 1, it appears that the compound is capable of permeating the cell membrane. One may then envision different scenarios: first, 3-OH HIPP could be metabolized within the cell or elsewhere localized. Second, 3-OH HIPP could be repressing CtBP2, but this repression is not sufficient to cause cell cytotoxicity. Accepting the possibility of this second option, one would infer then that 3-Cl HIPP and 4-Cl HIPP are CtBP-specific, but exhibit cytotoxicity through a number of different pathways. Based off luciferase data, these two molecules relieve Bik expression with concentrations that start to exude a cytotoxic effect. This leads us to believe that in large part the cytotoxicity is due to relief of CtBP expression. However, this does not eliminate off-target effects. One way to resolve this issue is to test localization of 3-OH HIPP. This could be performed by creating a radio-isotope labeled form of 3-OH HIPP and looking at localization of the molecule using autoradiography or electron microscopy. Outside of this, we can perform HPLC experiments as has been done for our lead HIPP analogues to look for loss of the hydroxyl group in water. We can also simply investigate whether any inhibition is seen in cell culture experiments, by looking at a profile of CtBP-regulated genes in the presence of 3-OH HIPP and other inhibitors.

Lessons learned from other CtBP-targeted therapies

Other laboratories have similarly seen the clinical relevance of CtBP as a therapeutic target and have begun to develop inhibitors by different mechanisms. Currently there are two

other compounds that have been reported for CtBP, and they were each found in very different ways. The first inhibitor was discovered by a reverse two-hybrid bacterial screen. They created fusion genes that were required for survival in selection media. These genes were only functional when CtBP dimerized. Through this screen, they looked for peptides that could impede cell growth as an indication of CtBP-dimer disruption. In this way a cyclic peptide was discovered that inhibits CtBP1 heterodimerization to CtBP2.⁹⁸ They verified this abrogation of dimerization by using an approach involving the Cos-7 cell line. Cos-7 cells are a unique cell line of fibroblast-like monkey kidney tissue that have a propensity to show a dependence of CtBP1 on CtBP2 for nuclear entry, due to the fact that CtBP2, but not CtBP1, has a nuclear localization signal. CtBP1 must heterodimerize with CtBP2 or it is constrained to the cytoplasm in over 60% of cells.³⁵ With transfection of a fluorescently labeled CtBP1 plasmid, one can see localization in the presence or absence of inhibitor. The peptide further induced micronuclei formation and caused significant cell cytotoxicity, as measured by colony formation assay at 50 μM concentration. The Cos-7 approach to showing disruption of dimerization is a clever cell-based assay, and the results seem promising for the peptide, but more work needs to be done. For example, this particular peptide has not been verified to be CtBP-specific, especially within cell-based cytotoxicity assays. Also, no work has been done yet to show relevance in *in vivo* models.

A third inhibitor of CtBP was identified using a process called an AlphaScreen that monitored the disruption of CtBP binding to E1A.⁹⁹ A hit was confirmed with fluorescence polarization, by using a fluorescein-labeled 14-mer E1A peptide to monitor the direct interaction between the two molecules. Through this process, a small molecule inhibitor was discovered that had weak substrate potential. It is not believed that it disrupts substrate binding, because in

their experiments MTOB addition did not impede E1A binding. Importantly, however, their inhibitor did alleviate repression of E-cadherin, a CtBP-regulated gene, by luciferase assay. One concern is that in this particular instance their CtBP inhibitor, NSC95397, has also been shown to suppress inflammation and to induce apoptosis by directly inhibiting other molecules including kinase phosphatase, MKP-1, Ca(2+)-binding protein, S100A4, kinase AKT, and several other proteins.¹⁰⁰⁻¹⁰³ The observation that this compound interferes with several specific pathways outside CtBP-interaction with binding partners prevents it from being carried forward as a targeted therapy. The study suggests that while assessing compounds for their ability to disrupt CtBP interaction with its binding partners is informative, it may not be the optimal first-tier screening assay.

Each of the different techniques for drug discovery has key advantages and potentially substantial drawbacks. Our insistence in using features that have hitherto been only found in CtBP-family proteins provides an advantage in helping to ensure that therapies can minimize off-target effects. Furthermore, it is critical that we work to validate compounds for CtBP-specific loss of viability.

Next generation targeting strategies

3-Cl HIPP and 4-Cl HIPP represent the best current small molecule compounds capable of disrupting CtBP activity. But for them to be therapeutically relevant, we would hope to decrease the concentration of the effective dose (the EC50 in MTT and colony formation assay) by orders of magnitude to low micromolar concentrations, thus improving the therapeutic index. Work is ongoing to both find better inhibitors using our rational design strategy and by studying combination therapies.

The Grossman lab has formed a collaboration with Dr. William Royer that allows us to use crystallographic research in order to visualize binding of potentially relevant inhibitors. When designing drugs with better binding affinity than these HIPP derivatives, it is important to note the space within the substrate-binding cleft⁸¹. To more fully displace the water network in HIPP (**Fig. 2_3G**) it may be possible to replace the imine (N-OH) and carboxylic acid moieties with larger groups that can increase bulkiness. There is still opportunity to do this, as our experiments did not directly probe larger moieties on the imine residue, and there was not an attempt to replace the hydroxyl of the carboxylic acid using the HIPP backbone. The intended end result of this would be total displacement of the water network which could help further increase CtBP-specificity.

When testing new compounds, we have shown that we have the basic schematic for a screening pipeline. CtBP enzymatic reactions formulate the first set of tests. As the second tier of screening, it would be useful to compare potential compounds using different dehydrogenases (e.g. lactate dehydrogenase), as was performed with the Cl-HIPP analogues,¹⁰⁴ to ensure that candidate inhibitors do not inhibit similar dehydrogenases. Third, determining a cytotoxic IC₅₀ is important to ensure the compound has clinical utility. Finally, it is important to determine CtBP-specificity, through luciferase assays and rescue experiments. While a sufficient rescue experiment has not been shown thus far, preliminary results of one strategy are presented in Chapter 4.

In addition to these initial screening tests, it will be useful to incorporate some of the assays that have been used to discover the other currently hypothesized CtBP-therapeutics. Each of these assays fills in a small piece of the puzzle. Does our inhibitor prevent dimerization? This can be assessed by immunofluorescence with the Cos-7 cell line and a GFP-tagged CtBP1

plasmid, showing the effect of CtBP2 heterodimerization by localization to the nucleus. Does a new inhibitor prevent CtBP from binding to a transcription factor, or other portion of its complex? A fluorescence polarization experiment could help to answer that question. Coupled with classical biologic techniques, such as immunoprecipitation, qPCR, and ChIP, we can identify the basic information needed to confidently take next generation inhibitors to mouse studies and further.

With good candidate inhibitors, we can start to test combination therapies. While HDAC inhibitor combination experiments were not demonstrated to be an effective strategy, there are several more avenues that can be pursued. One possible paradigm for combination therapy involves disrupting CtBP activity through metabolic regulation. We have previously discussed that CtBPs function as redox sensors, and that they have greater than 100-fold affinity for NADH over NAD⁺. The reduced form, NADH, is one of the products of glycolysis and can be interchangeably converted to NAD⁺ to carry out the metabolic processes of cancer cells. Due to a preference for glycolysis, tumors have a higher demand for glucose; thus the total pool of both NAD⁺ and NADH is higher in cancer cells than in most normal tissue. With this in mind, it is possible to take advantage of the aberrant metabolic environment of cancer cells at three points in the glycolytic pathway. First, it may be possible to lower the total glucose concentration in cells. Second, we can more specifically lower NAD concentrations within cancer cells. Third, one can alter the ratio of NAD⁺ to NADH. Each of these methods will have a plethora of effects, but all are predicted to disrupt both oncogenic function and CtBP's classical roles in cancer systems.

Therapies have been studied that target glycolysis at each of the aforementioned steps. For example, several drug compounds have been discovered as therapeutics that disrupt glucose

uptake. 2-deoxyglucose (2-DG) is a competitive inhibitor of glucose metabolism that becomes trapped following phosphorylation.¹⁰⁵ This in turn causes cellular ATP to be depleted, causing short supply of necessary glycolytic components. Since it is a competitive inhibitor, it must be used in high concentrations, as compared to intracellular glucose. It is observed to only partially reduce the access of glucose for glycolysis. 2-DG is not effective as a single agent *in vivo* but may be a reasonable and well-tolerated combinatory therapeutic. 3-Bromopyruvate (3-BrPA) inhibits hexokinase, the enzyme responsible for the first step of the glycolytic pathway, and is especially cytotoxic to cancer cells under hypoxic conditions or those involving mitochondrial defects. 3-BrPA is an order of magnitude more effective than 2-DG, but as an alkylating agent, it is also suspected to cause cell death by intercalating into DNA throughout the cancer genome. Some naturally derived glucose-uptake inhibitors have only been studied sparingly in clinical trials, including Genistein, a compound found in soybeans, and resveratrol, a phytoalexin found in grapes.¹⁰⁵⁻¹⁰⁷ Early studies suggest that these compounds may be good cancer preventative reagents, but much more work is needed to make any definitive claims. Resveratrol appears to be particularly attractive: in a variety of cancer cells, including breast and ovarian lines, it was shown to cause glucose deprivation, reduce lactate production, and retard cell proliferation.^{108,109}

In glycolysis, NAD⁺ can be synthesized from tryptophan or recycled from three salvage pathways, using nicotinamide, nicotinic acid, or nicotinamide riboside as precursors. Of these processes, the nicotinamide salvage pathway is the primary mechanism for NAD⁺ synthesis, and its rate limiting step is dictated by the action of an enzyme called nicotinamide phosphoribosyl transferase 1, or NAMPT.¹¹⁰ NAMPT inhibitors hold promise and have been interrogated in phase I clinical trials. In each clinical trial, dose-limiting toxicity was observed at low concentrations with late onset (>8 days) thrombocytopenia. For this reason NAMPT inhibitors

are not considered good single-target therapies, but may be useful at much lower concentrations in combination with CtBP-specific inhibitors.¹¹¹ Finally, research suggests that it may be possible to forcefully alter the NAD⁺/NADH ratio by adding NAD⁺ precursors to either cell or animal systems.¹¹² In one study, the specific addition of nicotinamide (NAM) increased the NAD⁺/NADH ratio. The treatment was able to prevent spontaneous breast cancer progression and metastasis, while increasing animal survival in mouse studies. This suggests that the NAD⁺/NADH balance is important for normal cancer processes. We propose that, in part, shifting NADH to its oxidized form will reduce CtBP activity, given its 100-fold lower preference for NAD⁺ and the necessity that CtBP has for NADH in order to dimerize and recruit transcriptional co-regulatory complexes. In effect, in conjunction with a CtBP-specific inhibitor that is targeted to the substrate binding domain, such as 4-Cl HIPP, we may be able to impair CtBP mechanisms by two independent targeting strategies: one disrupting NAD binding, and the other disrupting access to the CtBP substrate-binding domain.

Envisioning clinically relevant drug therapies

The ultimate goal of studying the therapeutic potential of cancer markers or oncogenes is to find targets that can be clinically relevant and make it to testing in clinical trials. We discussed in the rationale of Chapter 1 the properties that make CtBPs an ideal drug target. Included among them is the fact that CtBP1 and CtBP2 are clearly overexpressed in cancer tissue compared to normal adjacent tissue in several tumor types, suggesting that it is disease modifying. Several pathways have been elucidated in which it promotes cancer survival and growth. Disruption of CtBP complexes is known to cause apoptosis, independent of p53 status. We have developed crystal structures that have helped to identify very specific inhibitors. With these crystals and 3D-modeling techniques we can simulate other drug compounds. As we get

more data, the accuracy of these techniques will improve. We are able to test compounds using enzymatic assays. These assays are replicative and have proven informative in predicting the possible mechanism of action of substrate-localized CtBP-specific drugs. Further, it has been demonstrated in several instances, including our lab, that CtBP targeting by MTOB is a capable, albeit weak cancer therapeutic, providing incentive to continue down the road of drug discovery using similar inhibitors.^{30,70}

With all these findings that indicate that the design of CtBP-specific compounds may one day be a clinically useful cancer therapeutic strategy, it is necessary to be critical of potential pitfalls as well. There are a couple points to consider that could make CtBPs unfavorable as therapeutics that should be taken into consideration before moving drugs to clinical trials.⁷⁷ First, one should note that CtBPs have important physiological functions in the body, the most important of which is found in the brain. CtBPs are ubiquitously expressed in the brain at relatively robust levels.²¹ One may predict delivery of drugs should not be able to find access to the brain because of the partition of capillaries and endothelial cells that is known as the blood-brain barrier. Luckily for our purposes, the blood-brain barrier has historically provided a difficult obstacle for delivering drugs to the brain. However, since CtBPs have been implicated in normal cellular processes such as cell cycle and nuclear replication maintenance, off-target effects are something to consider^{30,113}. This point is intrinsic to the concept of the therapeutic index. Basically, the therapeutic index is measured by determining the concentration at which a drug is toxic in 50% of individuals divided by the concentration at which the compound is 50% effective. An ideal drug would have a very low effective dose and a very high toxic dose. In our lab we have seen that MTOB has a very high toxic dose in mouse models. In fact, we were not able to see any phenotypically negative effects at the highest dose, 750mg/kg, tested.

Unfortunately, the concentration used to determine an effective dose was also 750 mg/kg. For HIPP treated mice, experiments are currently underway to determine the effective concentration. However, we saw that concentrations at 500 mg/kg were cytotoxic to mice. As a good predictive model for low effective doses in animals, in a cell culture context we would hope to find drugs that are effective in nanomolar concentrations. Our best compounds thus far have EC50 concentrations in the high micromolar range.

A group from Bayer Healthcare©, in a paper discussing the attributes of good drug targets, suggested that an ideal target would include favorable prediction of side effects based on phenotypic data.⁷⁷ These models can include knockout mice or models containing mutations in the target of interest. Mouse models showed that CtBP1 null mice were viable, but that CtBP2 knockout mice died at embryonic day E10.5.¹⁹ These models seem disparaging, but in fact do not serve to predict phenotypes in adult onset cancers. A better model would include an inducible knockout of CtBP in various tissues, excluding the brain, in order to predict potential off-target drug effects. An inducible system would be necessary to ensure that pups are able to grow to adulthood before extinguishing CtBP expression.

Our discovery that 3-Cl HIPP and 4-Cl HIPP are better and more specific inhibitors than those previously shown is an encouraging next step. To increase the validity of these findings as a therapeutic there are a number of experiments that can give credence. For example, Dr. Mike Straza was able to demonstrate that for MTOB, cytotoxicity positively correlates with the degree of transformation of MEFs.⁷⁰ While these experiments have not been performed on newer generation compounds, it would provide an *in vitro* assay for demonstrating that modulating CtBP activity is less critical under more physiological conditions. It is hypothesized that similar

to MTOB, CtBP-specific compounds will show greater functionality in response to greater oncogenicity of cell lines.

Ultimately, the goal for a drug target is to be able to move it into the clinic. To envision one of these compounds becoming ready for clinical testing, we must identify optimal compounds from screening. This process is ongoing. Top compounds also need to be validated in mouse models. The results of *in vitro* studies are important for screening purposes, but negative results should not necessarily preclude further studies in animal models.

The results from **Figure 3_6** and **Figure 3_7** demonstrate this point. Based on cell culture data, CtBP-based therapies would not be effective in conjunction with gemcitabine. Mouse data contradicted this notion showing a clear synergistic effect caused by the combination of the two compounds. It is ethically unjustifiable to test all strategies in mouse models, but *in vitro* and *in vivo* results sometimes differ significantly. The best or most convincing targets should be considered for further development. Among these, one clear option from the data presented thus far is to test 4-Cl HIPP in conjunction with MTOB.

Finally, the specific target of these therapies should be considered. CtBP is upregulated in several forms of human cancer, including breast, colorectal, and ovarian. For the first set of mouse experiments, one can use immunodeficient xenograft mouse models inoculated with cancer cells of interest, but moving forward, studying a specific cancer type may prove beneficial to provide a proof of concept for clinical utility. The number of cancer mouse models are vast. In breast cancer, the models are known to better capitulate a human cancer-like phenotype, and there are models that share significant genomic similarity in tumor samples to the heterogeneity seen in human breast cancer tissue.¹¹⁴

Colon cancer models can be easily chemically induced (e.g. dimethylhydrazine and azoxymethane), and genetic models consist of APC^{min} models of Familial Adenomatous Polyposis (FAP) and Hereditary Non-polyposis Colorectal Cancer (HNPCC or Lynch syndrome).¹¹⁵ FAP patients have been shown to have increased concentrations of CtBP1 in adenomas, and so the APC^{min} and other models may be translatable to a patient population.⁶⁹ This altogether makes colon cancer models an attractive option for further study.

CtBPs are most often genomically duplicated in ovarian cancer (**Fig. 1_3**), and 83% of ovarian tumors overexpress CtBP2 versus normal adjacent tissue.⁷² Human ovarian cancer has a low 5-year survival rate of 46% (seer.cancer.gov) and is often difficult to treat. It may be worth pursuing CtBP-specific therapeutic treatments for ovarian cancer, but while mouse models are helpful, only a single, recently developed model exists that can recapitulate the most common type of ovarian tumor, high-grade serous adenocarcinoma.^{116,117} Coupled with reports that only 8% of animal model research in cancer is successfully translated to clinical trials,¹¹⁸ the ability to effectively study ovarian cancer is a challenge, albeit a worthwhile one. When envisioning the next steps for therapeutic development, there does not appear to be a clear single path forward, but several different models are worth further consideration.

Chapter 4: Early research into the biological relevance of the substrate domain in cancer cell systems, with a special emphasis on CtBP2 W324.

Rationale:

Up to this point, most of the progress made in our studies has come through manipulating the substrate binding domain of CtBP2. We have queried the mechanism whereby CtBPs may function; our hypothesis states that NADH binds CtBP to allow dimerization, and the major role of the substrate-binding domain is to help to disassemble the complex. However, only a few papers have demonstrably shown that substrate-binding in cell based assays or *in vivo* actually disrupts CtBP function, and not all studies seem to agree. To investigate substrate function, a series of point mutations were created using the crystal structure of MTOB and NAD⁺-bound CtBP to select for residues that may be informative. We intended to compare substrate-domain mutations with other regions of the protein that we know to be integral, explicitly mutations of the dimerization domain.

Previous studies have similarly sought to interrogate specific domains of the CtBP protein by creating point mutations and observing the effects. For example, an early study showed that mutating a dimerization residue, G183 in CtBP1 (G189 in CtBP2) was incapable of disrupting normal CtBP-mediated transcriptional repression in a Gal-4 reporter system.⁵³ Likewise, it found that a mutant of the catalytic domain, H315 (H321 in CtBP2), showed no difference in transcriptional repression based on both luciferase and Gal-4 reporter assays. They concluded that the dehydrogenase activity of CtBP was not necessary for transcriptional repression. Another study refuted this claim: G189 of mCtBP2 was found to be critical to allow NAD⁺ binding and dimerization.³³ Mutating the glycine to an alanine caused a significant

decrease in transcriptional repression potential in a Gal-4-tagged mCtBP2 assay.²⁷ This disruption of repression was caused by hindering CtBP dimerization. To a lesser extent, mutations of H321 and R272, two members of the CtBP catalytic triad, which are responsible for bringing substrate into close contact with NADH, disrupted transcriptional activity. A double mutant of G189R and R272L, completely disrupted CtBP-mediated repression.

Yet another study dissected mutations with regard to function.³⁹ A series of point mutations of the CtBP dimerization domain and a second series of point mutations of the catalytic domain were made. The mutations in the catalytic domain consist of the catalytic triad, G295, R266, H315, and a fourth mutation in D290 in CtBP1. This catalytic mutant disrupted binding to GST-tagged E1A and largely alleviated CtBP-mediated repression in a Gal4 reporter system, but it did not disrupt dimerization. By comparison, dimerization and NAD mutants dysregulated dimerization and had the gravest effects on disrupting CtBP regulation. This data was partially validated in drosophila where a G183E (corresponding to G189E in CtBP2) mutant completely abolished transcriptional repression activity as visualized in embryo development. However, H315Q (H321 in CtBP2) did not affect this repression.¹¹⁹ Finally, using a single point mutation in the histidine of the catalytic triad in dCtBP in drosophila, it was shown that catalytic disruption did not have a visible effect on development *in vivo*.⁴¹ It was capable of disrupting dehydrogenase activity in an *in vitro* system.

The published data, together, is somewhat contradictory, but most recent studies seem to suggest that the catalytic, or substrate-binding, domain of CtBP can disrupt transcription regulation and dehydrogenase activity. However, in studies that have been performed *in vivo* using drosophila model systems, it appears that mutations of the catalytic site do not have a biological effect. It should be noted that these systems look at catalytic function with regard to

drosophila development. Our research looks to study the biological effect of substrate mutants in cancer cell systems. Due to the vastly different systems and life events that are being studied, it is possible that the function of substrate binding in cancer cells differs immensely from that of drosophila development. The effort in this chapter also differs from previous publications that focused exclusively on the role of transcriptional function and dimerization. We endeavor to couple biochemical assays with biological assays to observe the result of disrupting substrate-binding on a variety of pro-oncogenic phenotypes.

Of particular interest to our study is the residue tryptophan 324 (W318 in CtBP1). It was discovered that this residue, found in the catalytic domain of CtBP, is unique among all dehydrogenases to CtBP-family proteins.^{39,79} The crystal structure shows that the residue is imperative for bringing substrate and NADH into close proximity. We have already started designing inhibitors that take advantage of an enhanced interaction with W324. In another study, it has been recognized that this unique moiety lies closely to, and may stabilize interaction with, NADH⁴⁵. As such, it was used for FRET (Fluorescence Resonance Energy Transfer) analysis to show that NADH has a lower K_d with CtBP than does NAD⁺, providing evidence that NADH is preferentially bound over NAD⁺. Recently, a study suggested that W318 was required for full dimerization of CtBP1 after NADH binding.⁴⁴ We hypothesize that this unique tryptophan may function as a sort of keystone for dehydrogenase and dimer function. We believe that mutation of this residue will cause phenotypic differences that prohibit numerous pro-oncogenic activities.

In this chapter, we perform a screen with several mutants to identify those that may be informative to carry over into cell based assays. We show the completion of one of these first assays, a cell migration, or scratch, assay. Further, we propose a system in which we can use

these mutations to study the specificity of new drug compounds. We show pilot data for this approach.

Materials and Methods:

Site-directed Mutagenesis and PCR:

Using a pet28a vector containing the truncated, 31-364 a.a., his tagged CtBP2 cDNA, site directed mutagenesis was performed using a protocol for single base pair substitutions. Briefly, non-overlapping primers were designed to create point mutations with the nucleotide substitution found in the forward primer followed by at least 10 nucleotides with perfect alignment. The reverse primer binds with perfect alignment, adjacent to the nucleotide substitution. Sample primers for W324G are shown below:

W324G Fw: 5-CTCACACTGCCCGGATACAGTGAGCAGGCGTC-3

W324G Rev: 5-GACGCCTGCTCACTGTATCCGGCAGTGTGAG-3

Primers were phosphorylated and PCR was run. The PCR mix and cycling conditions are as follows: 10ng template, 1uL of 10mM dNTPs, 10uL HF Phusion buffer, 0.5uM Fw Primer, 0.5uM Rev Primer, and water 50uL was added together, followed by addition of 1uL Phusion polymerase. Tubes were heated to 98C to denature, and a cycle consisting of denaturing for 10 seconds at 98C, annealing at 3 degrees above the melting temperature of the primers for 15 seconds, and elongation for 2.5 minutes. The cycle was continued for a total of 28 cycles. After PCR, the products were ligated and transformed into DH5a chemical competent cells and raised on a kanamycin background. Successful mutants were confirmed by sequencing.

Informative mutations, as determined by NADH reaction curves, were cut by restriction digestion and cloned into the full-length protein, which has a C-terminal V5 tag, in the pcDNA3.1 vector. CtBP2 cDNA contains single EcoRI and EcoNI restriction sites flanking the mutated region that are not found in the pcDNA vector. Unidirectional clones were generated by excising the insert with EcoRI and EcoNI restriction enzymes and then by re-ligating the mutation into the full-length protein using T4 DNA ligase. Successful ligation and transformation were verified by gel electrophoresis and sequencing. Dr. Priyadarshan Damle created, or assisted in the cloning of, several mutations presented in this work. Point mutants include: normal, (wildtype, WT) CtBP2, H83Q, R103K, V111I, R157A, G189A, R272A, R272K, H321A, H321Q, W324A, W324H, W324F, W324G, and Y325A.

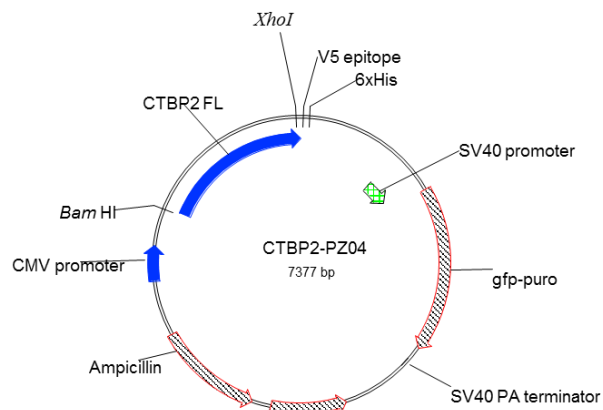
Creation of Silent Backbone CtBP and Integration into a PZ04 vector:

A previous member of the Grossman lab designed a custom CtBP2 siRNA. Using the backbone of that siRNA we created a series of silent mutations:

siRNA for wtCtBP2:GCGCCTTGGTCAGTAATAG

Silent Mutant CtBP: GCGCCATGGTCTTGTTATTG

Further, Dr. Priyadarshan Damle in the Grossman lab created a vector to create stable mutant cell lines and visualize transfection efficiency. The plasmid (map below) contains restriction sites for the transfer of CtBP2 from pcDNA using restriction enzymes for excision and re-ligation. CtBP2 is expressed under a CMV promoter. A second gene, GFP-puro is expressed under the SV40 promoter. CtBP2 mutants created in pcDNA3.1 were transferred through restriction digestion and ligation with shared restriction sites of the multiple cloning sites, Bam HI and XhoI.



Reaction Curves and k_{cat}/k_m Determination:

CtBP2 protein (a.a. 31-364) was purified, as described in the method section of Chapter 2. After purification, the concentration of each mutant was determined by BSA assay and purity was assessed by Coomassie stain. For reaction curves, 150 μ M final NADH and 50 μ M MTOB were added together with 18 μ g total CtBP2. Runs were read in triplicate and absorbance was measured at $A=340\text{nm}$ every 30 seconds for 1 hour. Data represents the average of 3 to 9 reads.

K_{cat}/k_m is a measure of the efficiency of an enzyme, where k_{cat} represents the number of substrate molecules the enzyme can turn over per second time, and K_m represents the affinity for substrate, defined as the substrate concentration at half the maximum rate of the reaction. It is defined by the equation $V = k_{cat}[E]_t[S]/(K_m + [S])$, where V = enzyme activity, $[E]_t$ is total enzyme binding sites for a substrate, $[S]$ = substrate concentration. For this assay, CtBP protein was used at a concentration of 20 μ g/ml, which is the equivalent of 986.2nM. Since we assume that each molecule of CtBP only has one binding site for MTOB, $[E]_t$ is equal to 986.2nM. The enzyme activity was determined by calculating the initial velocity of the enzyme by keeping levels of NADH constant and in far excess, and varying the concentration of substrate with each run. NADH was added at a final concentration of 150 μ M. The different concentrations of

MTOB were 48, 36, 24, 12, 8, 4, 2, 1, and 0 μM . With the enzyme concentration, Graphpad Prism® was used to calculate k_{cat} and K_m , reported in this case as $\mu\text{M}/\text{sec}$.

Assays were performed by adding all components, except enzyme, together and allowing them to incubate. CtBP was then added, quickly mixed by manual pipetting and immediately placed in the spectrophotometer at $A=340\text{nm}$. The assay was measured for five minutes and initial velocity was calculated by calculating the slope from the first linear phase of the assay. Assays were performed with at least three measures to attain a value.

Transfections:

Transfections were performed using lipofectamine. Briefly, two tubes containing 160 μL Gibco® OptiMem™ were used for each condition. Purified plasmid DNA was added to 1 tube and Lipofectamine® 2000 (Thermo Fisher) was added at a ratio of 1.5:1, μL Lipofectamine:ug DNA. Tubes were incubated for 5 minutes and then combined. After a further 20-minute incubation, they were evenly added to 6cm plates containing cells at 50% confluency in media lacking antibiotics. Cells were harvested at the time points indicated.

Stable Cell Line Generation:

HCT116, p53^{-/-} or MDA-MB-231 cell lines were transfected with pZ04 plasmids containing vectors for: empty vector, wild type CtBP2, CtBP2 mutants G189A, R157K, R272A, H321Q, Y325A, W324G, W324F, H83Q, and R103A. After 48 hours, they were selected for with 8 μL puromycin 50mg/mL. Cells were expanded until reaching 80% confluency and then Fluorescence activated cell sorting (FACS) was used to select for high GFP-expressing cells. Extra aliquots were frozen, and actively dividing plates were maintained in media containing 4 μL of 50mg/mL puromycin per 10cm plate.

Cell Migration Assays:

For Migration Assay, equal number of MDA-MB 231 cells were plated at 50% confluency in a six-well dish. The estimated number of cells plated was approximately 5×10^5 . Cells were then transfected as above with 3ug DNA. 48-hours post-transfection, a small wound was scratched into the confluent cell monolayer to form a cross pattern in each well. Pictures were taken in 2-hour increments until a total of 8 hours had passed. Images were captured using an electron microscope at 4x at the intersection of the cross. They were used to compare and quantify the percent closure of the wound, measuring the 'free' area using a free source scratch assay macro in Image J software. Each mutant was measured in triplicate for each independent experiment. Data was measured as a rate of migration per hour, and analyzed by One-way ANOVA followed by Tukey's post-test.

Western Blot Analysis:

Cell cultures for western blot analysis were rinsed in cold PBS and harvested in RIPA buffer, pH 7.2 containing protease inhibitors using a cell scraper. The lysate was rotated at 4° C for 20 minutes and then the cell membrane was removed by centrifugation at 12000g for 20 minutes at 4° C. Lysates were quantified against BSA protein assay standards and 20ug was loaded onto a 3-12% gradient NuPage® Bis-Tris gel. Western blots were run and transferred using the Invitrogen NuPage® Novex ® Gel System on to a nitrocellulose membrane. The transfer membrane was blocked with 0.5% Casein and probed with either goat-polyclonal (Santa Cruz) CtBP2, monoclonal CtBP2 (BD Biosciences), or V5-antibodies (Abcam). B-actin was used as a loading control. Membranes were rinsed with TBST (Tris buffered saline with Tween 20). Fluorescent antibodies were used for visualization of the blot by the Licor® Odyssey scanner.

Drug Resistance Assays:

To measure any differences in drug tolerance in cell lines containing mutants for our plasmids, CtBP2 null, HCT116 p53^{-/-} cells were used. These cells were previously constructed using the CRISPR/cas9 system by Dr. Priyadarshan Damle, and were verified by sequencing. CtBP2 was disrupted by two different frame shift mutations and western blot showed complete loss of CtBP2 product. One cell line, called CtBP2^{-/-} 6.1 was transfected with pZ04 plasmids at a concentration of 1 µg DNA per 100,000 cells and 1000 cells per well plated in a 96 well plate. After 48 hours, the cells were treated with roughly EC50 concentrations of drug as determined by values generated in **Table 1** (4mM HIPP, 1mM 4-Cl HIPP, 1mM 2-OMe HIPP, 0.5% DMSO). After a further 48-hour incubation, cells were treated for MTT assay, as described in the methods of Chapter 2. Values were normalized to a background frequency and then mutants were normalized to DMSO controls to account for variability due to transfection differences.

Results:

Evaluation of CtBP substrate-domain mutants by enzymatic assay

Using the crystal structure of MTOB-bound CtBP to analyze the substrate-binding pocket, several amino acids were predicted to play a role in the substrate-binding domain. These residues include: R272, H321, H83, R103, V111, R157, G189, W324, and Y325.

R272 and H321 represent two of the three members of the catalytic triad, a conserved group of amino acids found across a variety of different enzymes. The catalytic triad is found at the active site and provides support for dehydrogenase function. H83 and R103 are residues that lie in close proximity to MTOB, with favorable charges for hydrogen bonding interactions. V111 is a residue that differs between CtBP2 and CtBP1 (an isoleucine is found at this site); it is not predicted to have any role significant for substrate-specificity. R157 and G189 are amino acids found within the dehydrogenase domain, and are considered dimerization mutants. G189 especially is well described as the middle glycine of a three glycine structure (GxGxxG) which forms a conserved dinucleotide binding motif.⁴⁴ W324, as has already been described, is a major focus of our research and is considered to be an integral component of CtBP-substrate specificity. Y325 is found adjacent to W324. It was included because it was predicted that the tyrosine molecule could provide residual support for the tryptophan.

Mutations of each of these residues were designed to disrupt the normal function of the amino acid residue. For example, H321 was converted to a glutamine (Q), which has a similarly sized branched chain and is neutrally charged and hydrophobic, in contrast to histidine (H) which is a moderate amino acid (neither strongly hydrophobic nor hydrophilic) and positively charged. Arginine (R) residues were mutated to lysines (K), a similarly positively charged, hydrophilic amino acid that has a single amino group at the end of its chain, instead of the more extended

guanadino chain typical of arginine. To test for the effect of complete disruption, many amino acids were substituted with an alanine (A) molecule, whose chain consists simply of a methyl group. Tryptophan (W) 324, as the primary target of our investigation, was replaced with a phenylalanine (F) to see if the phenyl ring moiety is the essential component of the side chain. It was also replaced with an alanine and glycine (G). Glycine residues are capable of inducing greater change than other amino acids due to the lack of a side chain, which allows for significant alterations in protein folding.

Synthesis of point mutations was accomplished with site-directed PCR techniques. Protein was expressed for each mutant and used for NADH disappearance assays in order to determine which mutations may be the most informative to carry forward to further studies. Reaction curves were created, showing the enzymatic activity of each mutant carried out over a period of one hour. Several of the more informative mutants are shown in **Figure 4_1**. The change in absorbance over time was normalized to show how each enzyme compared as a proportion of the maximum conversion of NADH to NAD⁺ in wild type CtBP2.

In line with our predictions, W324F and V111I had reaction curves that mirrored that of wild type CtBP2 protein. The implication of this suggests that a W324F mutant should function similarly to wild type in other assays, and also indicates that the key portion of the tryptophan residue for normal function is the phenol ring. V111I showed that neither of these amino acid substitutions at a.a. 111, while different between the two CtBP family members, contribute to the role of dehydrogenase activity.

The other W324 mutations tested, W324G and W324A showed a severe impairment of enzymatic function. As far as can be seen, W324G is enzymatically dead. Indeed, compared to the mutant G189A, which impedes NADH binding, W324G is very similar. W324A appears to

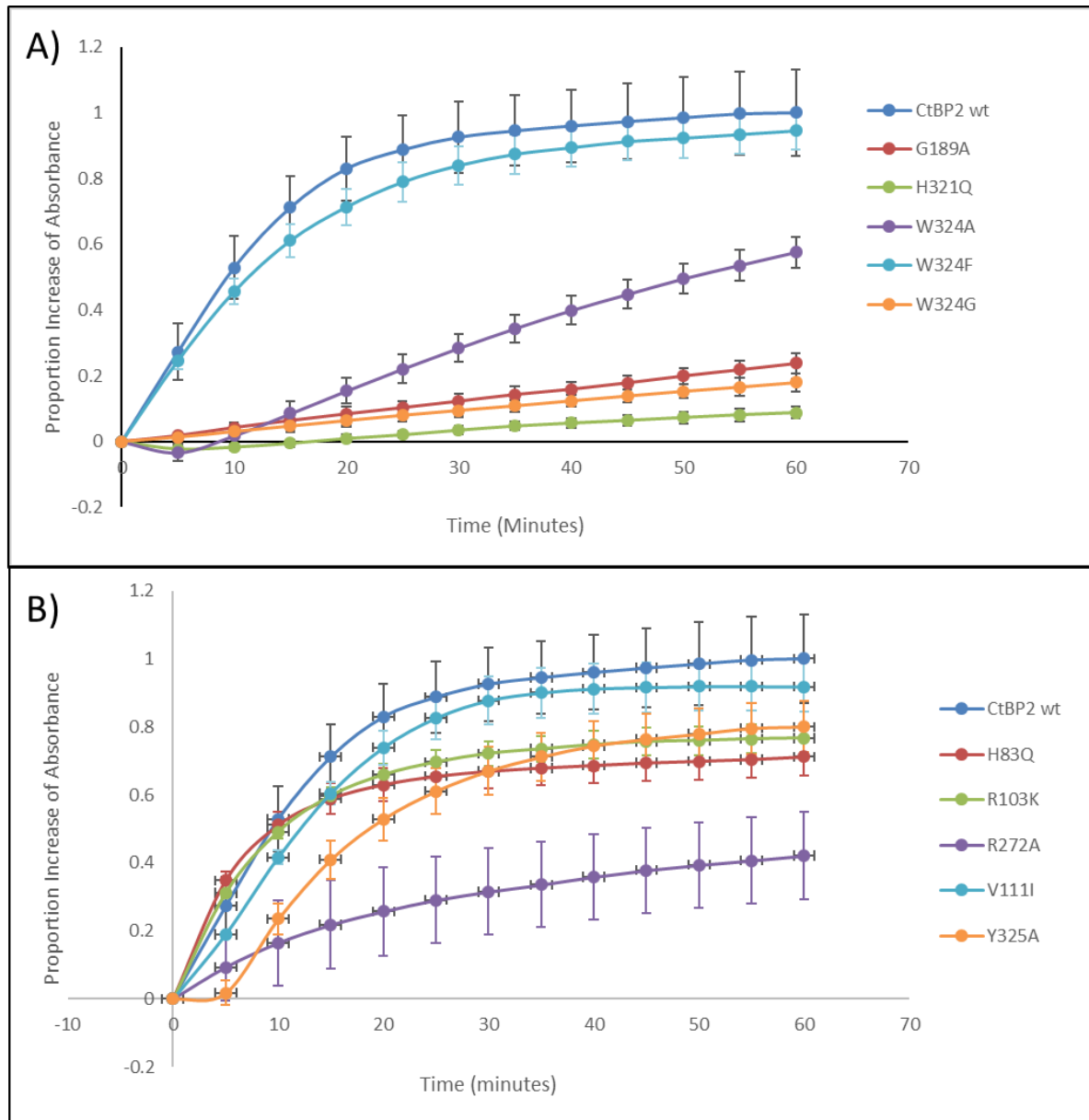


Figure 4_1. Reaction curves for wildtype CtBP2 protein and several mutants. NADH disappearance assays were performed over a time course of 1 hour. Several mutants were assessed including several that were carried forward into biological assays **A)** and that were selected for interesting properties **B)**. Error bars represent the 95% confidence interval and all values were measured 3-9 times.

have very slow residual activity. Mutations of the catalytic triad, R272A and H321Q, showed almost no enzymatic activity. The other amino acids of interest, H83Q and R103A, displayed an intermediate level of activity. Each appeared to have an initial change in absorbance similar to wild type protein, but peaked at a lower level of total absorbance. This may be attributed to a possible difference in protein stability. The cause for this difference was not investigated further, but the results imply that H83 and R103 do play a role in CtBP regulation. Finally, the mutant Y325A appears to trend toward reaching peak absorbance levels, but has an unknown delay in velocity.

Reaction curve data, while a good visual indicator of the trends seen in enzymes, do not give a quantitative measurement with regard to the efficiency in which an enzyme can catalyze a reaction. But if one were to take several reaction curves and measure the velocity of the rate of the reaction at the beginning, it would be possible to compile all the data to generate a number of true enzyme efficiency. This number is referred to as the constant k_{cat}/k_m (a more detailed explanation of this value and how it is calculated can be found in the methods of this chapter). We endeavored to determine this value for wild type CtBP2 protein and W324A, W324F, H321Q, R272K, and H83Q mutants. We generated this value for only three: wildtype CtBP2 had a $k_{cat}/k_m = 0.269 \mu\text{M}^{-1}\text{min}^{-1}$, W324F $k_{cat}/k_m = 0.268 \mu\text{M}^{-1}\text{min}^{-1}$, and H83Q $k_{cat}/k_m = 0.182 \mu\text{M}^{-1}\text{min}^{-1}$. Unfortunately, based on model fitting provided by Graphpad Prism software, the goodness of fit was measured as $R^2 = 0.65, 0.29, \text{ and } 0.28$ respectively. For other mutants no values were able to be determined, in part because either the initial velocity was too slow to measure within the framework ($t=5$ minutes) of our assay, or because activity was altogether dead. Despite difficulty in generating a true constant value to describe the enzymatic activity of CtBP enzyme and its mutants, we have demonstrated that the reaction curves give an accurate

indication of true enzyme potential or abrogation. Based on the information generated by enzymatic analysis, W324A, W324F, W324G, H321Q, and G189A were taken forward for evaluation in biological assays.

Early biological evaluation of CtBP mutants

To date, no studies have been performed to look at the phenotypic effect of CtBP-substrate domain mutants on cancer cell systems. Attempts to study pro-oncogenic differences in cell culture were met initially with difficulty in the Grossman lab, due to two basic issues. The first is that CtBP appears to be tightly regulated. Anecdotally, members of the lab have not been able to overexpress exogenous CtBP much more than twice the amount of endogenous CtBP, and elevated CtBP rapidly decreases with time. Second, different mutations transfected with slightly different efficiencies. Those mutants that seem most disruptive appear to have the best incorporation of CtBP into cells, perhaps due to the fact that since they may be non-functional or promote novel phenotypes, they can evade normal CtBP regulation. A further discussion of interventions that have been attempted in order to resolve these concerns is found in **Appendix II**.

We first set out to discover if CtBP2 mutants were capable of inducing differences in the migratory capability of cancer cells. CtBP has been demonstrated to both inhibit genes such as E-cadherin and activate genes like Tiam1 to promote migratory and invasive phenotypes^{31,54}. Cell migration scratch assays were performed in MDA-MB-231 cells, a highly invasive and metastatic breast cancer line. Cells were transfected with each of our mutants and allowed to grow to a confluent monolayer, at which point a wound was made on the plate and the migration of cells into the open space was measured (**Fig. 4_2**).

Migration rates indicate that CtBP2 overexpression is able to significantly increase the rate of migration in cell culture, as was expected. W324F mutant migration is indistinguishable from that of wild type, reemphasizing that the phenol group of tryptophan is imperative for proper function. Both W324G and W324A mutants had migration rates that were the same as an empty vector transfected control. Similar rates of migration were seen with H321Q and G189A. The similarity between rates of migration in these mutants compared to empty vector suggests that there is no advantage gained from an enzymatically non-functional mutant. On the other hand, there was no evidence of a dominant negative phenotype. From this first assay, catalytic domain residues seem to play an integral role for migratory function. Future experiments will look to further define the consequences of CtBP mutation on other phenotypes. Specifically, MTT assay, cell count monitoring, clonogenic and other assays will measure cell proliferation and viability differences. Invasion assays will take the results of migration data a step further. Additionally, mutants can be used to assess resistance to cytotoxic insults.

In the previous chapter, luciferase results showed that CtBP-specific inhibitors appeared to induce Bik expression. While this is a good first step in verifying that CtBP-inhibitors actually cause cytotoxicity through CtBP-specific mechanisms, one of the best ways to verify the results is through rescue assays. In these assays, addition of higher amounts of CtBP should start to ameliorate the percentage of death, preferably in a dose dependent manner. We hypothesized that we could similarly see this effect with mutant proteins. Specifically, we conjectured that if mutants had at least remedial function, but had a decreased affinity for inhibitor binding, they would be able to partially rescue CtBP-mediated apoptosis. A pilot study was performed to show proof of the concept using CtBP2-null cells generated by CRISPR (**Fig. 4_3**). The

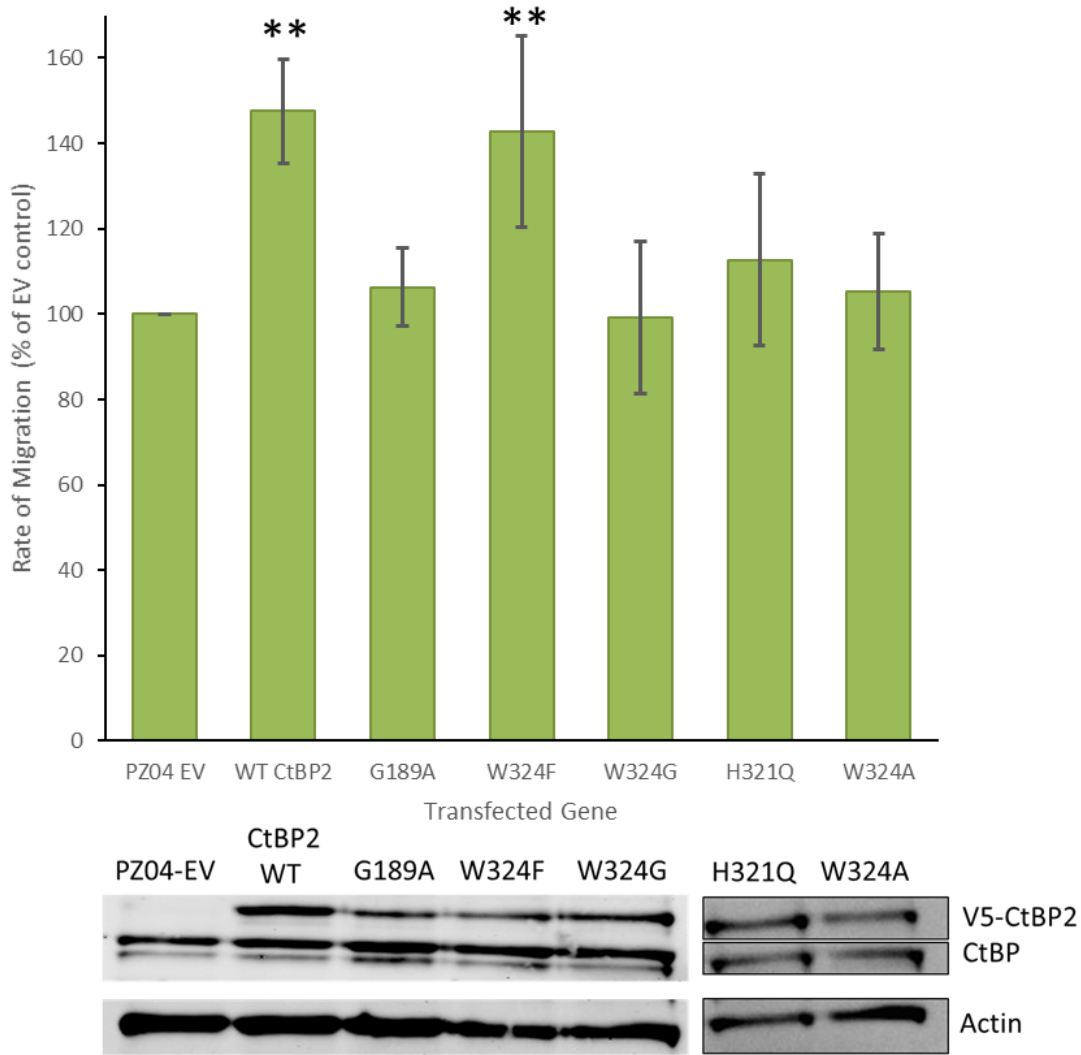


Figure 4_2. Cell migration is dependent upon functional CtBP2. An empty vector (EV), CtBP2, or its mutants were transiently transfected into MDA-MB-231 cell lines and a wound was created. The rate of migration into the wound was measured after 8 hours (above). A Western blot shows lysates recovered from the assay showing representative transfection of each mutant (V5-CtBP2) compared to endogenous levels. Actin serves as a loading control. *= $p < 0.01$ vs. PZ04-EV. Data represents the average of $n \geq 3$ independent experiments, measured in triplicate. Error bars represent standard deviation. The data in this figure was generated by Zaid Nawaz.

experiment makes the assumption that HIPP and 4-Cl HIPP, in reality induce cell death in part through relief of CtBP-repression. We expect that relevant ‘rescue’ mutants would show increased survival over an empty vector control or wild type exogenous CtBP in the presence of these two drugs. We further expect that in the presence of non-CtBP specific inhibitors, like 2-OMe HIPP, the proliferative advantage would disappear.

Looking at a spectrum of CtBP2 mutants in the presence of HIPP (**Fig. 4_3**) we see that several of the compounds had increased viability compared to the empty vector control. By comparison, three of those mutants, G189A, R103A, and Y325A continued to show increased cell survival in the presence of the non-CtBP-specific inhibitor 2-OMe HIPP (**Fig 4_3C**). This suggests that the mutants confer a proliferative advantage that is not dependent on the mode of cell death. Presuming similar trends are seen with replication experiments, they should be disqualified as rescue mutants. 4-Cl HIPP serves to validate results seen in the HIPP experiment and shows that three mutants remain as candidates (**Fig. 4_3B**). W324G very subtly expresses the pattern expected for a rescue mutant, but at levels that may be hard to quantify in future assays. R272A and H83Q show almost identical expression patterns above all other valid candidates, with roughly 50% increased survival in the presence of HIPP and 4-Cl HIPP and no difference in survival with 2-OMe HIPP, compared to empty vector. Based on our hypothesis, R272A and H83Q meet the criteria of potential rescue mutants.

This experiment is a pilot and so results cannot produce general conclusions, but it does provide encouragement that using substrate-binding mutants as rescue mutants is a technique that is worth pursuing in further detail. Efforts are underway to do so. Together we have shown that CtBP2 mutants of the substrate-binding domain are important for cell migration and that mutants

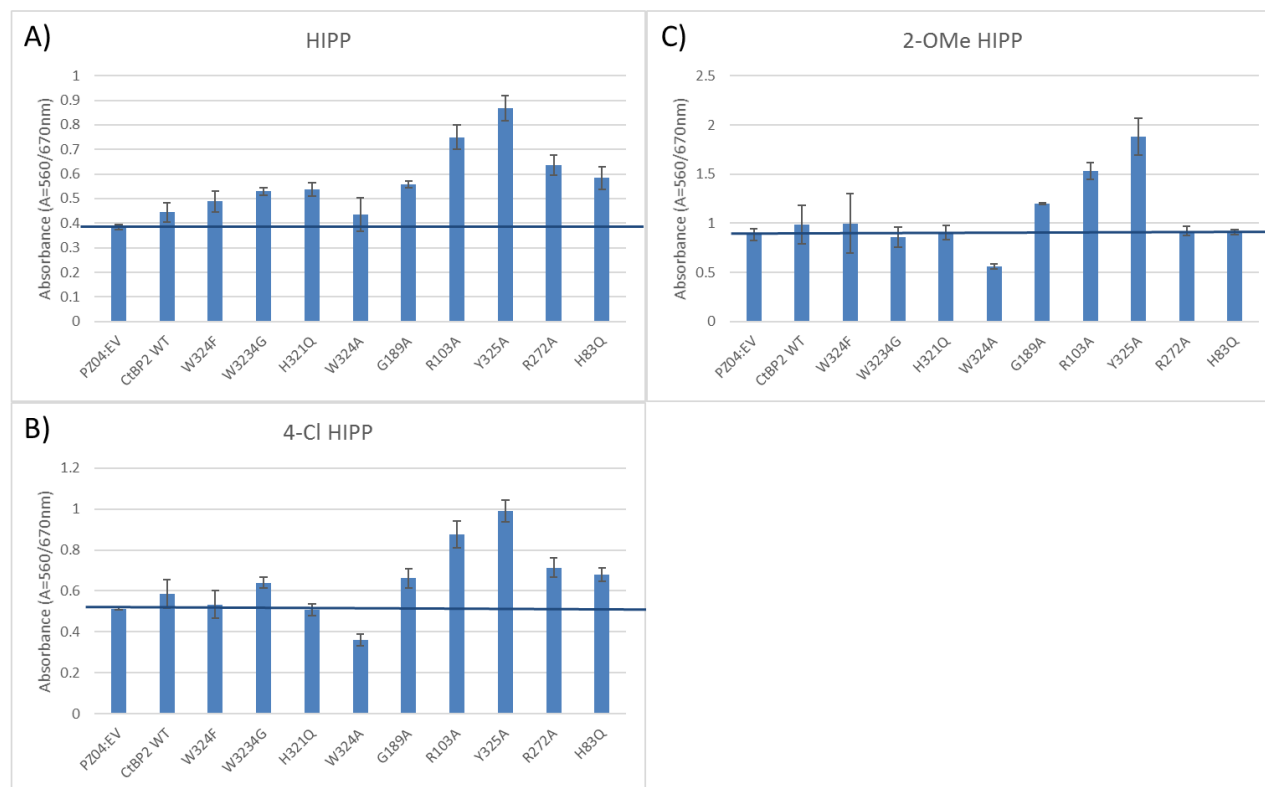


Figure 4_3. A pilot study of CtBP2 mutant resistance to CtBP-specific compounds. CtBP2 wild type, mutants, or empty vector were transfected into CRISPR-generated CtBP2 null HCT116 p53^{-/-} cells and incubated 48 hours before drug treatment with **A)** 4mM HIPP, **B)** 1mM 4-Cl HIPP, and **C)** 1mM 2-OMe HIPP. Candidate mutants for rescue experiments are those that have higher viability than EV controls with HIPP and 4-Cl HIPP, but not 2-OMe HIPP. Blue horizontal lines show the level of viability in EV transfected cells. n=1 independent experiment. Error bars represent standard deviation from triplicate well measures.

may provide a mechanism for studying further CtBP-specific therapeutics, by creating an easily interrogated rescue assay.

Discussion and Future Directions:

Mutants were generated to assess the role of substrate-binding in CtBP2 in cancer biological processes in order to be able to understand the physiological function of substrate for CtBP. Several mutants were shown to inhibit enzymatic function, including W324A, W324G, H321Q, R272A, and G189A. With the mutants that were subsequently carried forward into cell migration experiments, the level of enzymatic function seems to correlate with biological function. W324F mutants had nearly identical enzymatic function and migration rates as wild type CtBP. This provides confirmation that the phenyl ring is important for normal function and is encouraging for the further development of inhibitors that take advantage of close association with this unique tryptophan through pi-pi stacking. We further showed that W324G and W324A mutants were non-functional in cell migration assays; they neither increased nor decreased rates of wound closure as compared to empty vector. This provides evidence that W324 may actually serve as a key residue for CtBP function. G189A, a mutation that inhibits NADH binding and impedes subsequent CtBP dimerization, proved to be similarly non-functional. Interestingly, H321Q also showed limited activity in cell migration assays. This contrasts with the results described in drosophila development data.^{41,119} In both of those previous studies, H315 (analogous to H321) was found to not be necessary for development *in vivo*. We found that H321 impedes the normal function of CtBP in cancer cells to promote cell migration. One assay is insufficient to infer much with regard to biological function and so other assays are needed. To focus on key pathways involved with CtBP-mediated oncogenesis, we propose including

assays to measure cell invasion and proliferation, cell cycle regulation, and resistance to cell death.

Building upon this, in order to understand CtBP substrate binding by way of mutant studies, it is necessary to perform many of the transcription based assays that have been discussed previously. Especially with CRISPR produced CtBP2-null cells, we will be able to determine differences in transcription caused by even subtle alterations from mutant phenotypes. We have at our disposal functional luciferase assays and a number of target genes that have been validated for qPCR analysis. This, along with upcoming ChIP results, will permit the Grossman lab to, within the next couple of years, have a developed understanding of the role of the CtBP-substrate binding pocket.

A goal of understanding substrate-binding is to be able to have a firmer appreciation of the mechanisms of CtBP so that we can create therapeutics that have limited unintended consequences. In the process of developing drugs, it is important to create a rescue assay that shows whether inhibitors are CtBP specific in causing apoptosis. We proposed a model wherein we use a semi-functional mutant of CtBP to validate CtBP-specificity. This would be especially useful due to the difficulty of maintaining upregulated CtBP, which makes rescue assay development tedious. Of all the mutants screened, H83Q is particularly interesting. It shows relatively normal enzymatic function, and was able to cause increased cell survival versus controls in an apparently CtBP-specific manner. The next biological assays will include H83Q and expand on the mutants that hitherto have been tested.

Chapter 5. Final Conclusions

CtBP2 is one of two members of the CtBP-family transcriptional co-regulators that play an essential role in development. Increasingly CtBPs have been indicated as contributors to cancer development and progression. In this dissertation we delineate the characteristics that make CtBPs good targets for drug therapy. In part, CtBPs exhibit therapeutic potential because they have an intrinsic enzyme activity that can be utilized for drug screening. In addition, they have been shown to promote several pro-oncogenic processes, such as EMT, cell migration and invasion, cell proliferation, regulation of metabolism, and cell cycle regulation. CtBPs are more highly overexpressed in a number of patient tumor samples than in corresponding normal tissue, implicating their role as either a marker or a driver of cancer progression. This overexpression is stimulated by increased NADH concentrations, as seen in hypoxic conditions and through preferential glycolysis, seen in cancer via the Warburg effect. CtBPs can be sequestered and targeted for proteasomal degradation, ultimately leading to apoptosis due to de-repression of tumor suppressors, such as BH3-only apoptotic proteins Bik, Bim, and Bax. This apoptotic effect is seen regardless of the p53 status of the cancer tissue. MTOB treatment has served as a proof of principle that CtBP targeting has the potential to be therapeutically beneficial. In MEFs that are mutated to reflect various degrees of oncogenic transformation, MTOB cytotoxicity correlates positively with oncogenic potential.

The data presented in this work shows the development of a series of better inhibitors than MTOB. MTOB has limitations as a cancer therapeutic. At low levels, it mildly increases cell proliferation and it requires high concentrations to switch from functioning as a substrate, used for CtBP dehydrogenase activity, to an inhibitor. A high-throughput screen of 30,000

compounds failed to indicate a better class of inhibitors but inspired development of transition state mimetics (**Fig 2_1**). The crystal structure of MTOB-bound CtBP indicated that we could create tighter binding by maximizing the association with a tryptophan residue that is unique to the substrate-binding domain of CtBPs, among all other dehydrogenases. We used information gleaned from these two avenues of research to increase pi-pi stacking by creating a phenyl ring to replace the thiol residue of MTOB. Further, the ketone on C-2 that normally undergoes hydride transfer with NADH was replaced with a non-reducible imine molecule (**Fig. 2_2B**). This formed 2-hydroxy-3-imino-phenylpropionic acid, or HIPP, a >1000-fold better inhibitor in enzymatic NADH turnover assays. Determination of a constant of inhibition for HIPP, K_i , indicated an unusual pattern of seemingly non-competitive inhibition (**Fig. 2_4**). An investigation of the literature and simulation studies suggest that HIPP can form a ternary abortive complex, wherein it can bind to CtBP after the redox reaction between MTOB and NADH. It interacts after the release of MTHB, but before the release of NAD^+ (**Fig. 2_5**). This model also proposes that CtBP's normal mechanism of dehydrogenase function involves an ordered bi-substrate reaction, where NADH is required to bind first for a conformational change to occur (domain closure) that allows CtBP dimerization. This is followed by MTOB binding which leads to a redox reaction and release of both substrates; however, MTOB is equally capable of binding first, and as a result can prevent NADH binding and enzyme accessibility. This was further demonstrated by illustrating that MTOB causes substrate-inhibition at concentrations in excess of NADH (**Fig. 2_4D**). The close concordance between the generated data and simulations (**Appendix I**) confirms this as a valid possible mechanism. Current and future experiments are needed to validate this hypothesis. This is being done through pre-incubation experiments wherein HIPP and MTOB are added before NADH to test disruption,

and analyzed by GST pulldown experiments, to look at dimerization, and by NADH disappearance assay. Analytical ultracentrifugation is also used to determine if HIPP addition can push CtBP from a dimer toward a monomeric state. Knowing the state in which CtBP is bound by inhibitor will help validate the usefulness of HIPP and other compounds as drugs that can isolate the CtBP complex from the promoter regions of tumor suppressor genes.

HIPP was tested in cell viability assays and not shown to be a substantially better cytotoxic agent than MTOB (**Fig. 3_1**). In order to improve therapeutic index, we hope to find an agent that is specific and can induce cell death at nanomolar concentrations. By deconstructing regions of phenylpyruvate (**Fig. 3_2**), we found that each element studied was important for HIPP-enhanced binding. Moieties were substituted around the phenyl ring of HIPP (**Fig. 3_3**). Two inhibitors, 3-Cl HIPP and 4-Cl HIPP were shown to be better inhibitors and roughly 4-fold more effective cytotoxic agents (cytotoxic EC₅₀ values of 0.85mM and 1.74mM respectively, **Table 1**) than HIPP. Among others, 3-OH HIPP and 2-OMe HIPP were shown to be non-effective drug compounds that served as controls for other experiments. 3-OH HIPP was shown to be an inhibitor in enzymatic reactions but failed to induce cytotoxicity. Conversely, 2-OMe HIPP is extremely cytotoxic but not CtBP-specific (EC₅₀=1.24 mM). With these drugs, we saw that 3-Cl HIPP and 4-Cl HIPP relieved Bik promoter repression in a luciferase assay, a process presumed to occur through displacement of CtBP. This correlated with CtBP-specificity and cytotoxicity, as 3-Cl HIPP and 4-Cl HIPP were the only compounds that showed this response (**Fig. 3_5**).

Combination drug regimens were studied, and it was found that chemotherapeutic reagents did not exhibit additive or synergistic cytotoxicity in cancer cells (**Fig. 3_6**), but HIPP and gemcitabine proved to be a synergistic drug combination in mice (**Fig. 3_7**). Additionally,

HDAC inhibitors in combination with HIPP did not increase cellular cytotoxicity (**Fig. 3_8**). In further combination therapies, 4-Cl HIPP, but not 3-Cl HIPP, showed an additive effect with MTOB, decreasing the effective concentration for each by 15-fold and 4-fold respectively (**Fig. 3_9**). Work is underway to replicate these experiments, and mice studies have begun in order to test the maximum tolerated dose of 4-Cl HIPP. From this, we will have a starting point from which to perform a variety of studies to determine the ideal therapeutic regimen *in vivo* with the current molecules and tools at our disposal.

Finally, to enhance our understanding of the impact of using substrate-binding inhibitors, we created a series of point mutations in CtBP2. Mutations of some key substrate-binding residues, such as R272A and H321Q of the catalytic triad, were enzymatically dead (**Fig. 4_1**). H321Q was further studied in a cell migration assay and found to be nonfunctional. We made a special emphasis to study W324 mutants, as we predicted that this unique tryptophan served as a ‘keystone’ residue that permitted NADH-binding, dimerization, and substrate-binding. W324F mutation did not show a difference in enzymatic activity or cell migration ability (**Fig. 4_2**). More severe mutations, W324A and W324G, were enzymatically impaired and, in migration assays, nonfunctional. A scheme was proposed to create a rescue assay using substrate-binding deficient mutants as a means to study CtBP-specificity for future inhibitors (**Fig. 4_3**).

Taken as a whole, this data takes a step forward in the development of CtBP-specific therapeutics. It is hoped that this work contributes to the eventual development of a therapeutic strategy that can be used in clinical trials. Further efforts are necessary, and will largely be driven by the results of mouse studies. We are beginning to study one of our best next generation lead compounds, 4-Cl HIPP, in mouse models. In addition, there is room to find other inhibitors. Two water molecules co-occupy the substrate-binding domain pocket with

HIPP; potential development of more specific compounds could be designed to further fill this cavity. Evaluation of novel compounds can be studied through a pipeline of enzymatic inhibition, cytotoxicity, regulation of CtBP target genes and gene promoters, and cytotoxic resistance in rescue assays, as has been laid out in this manuscript.

As results have been uncovered, the repercussions of this work have branched tremendously. Collaboration has increased within the Grossman lab and throughout VCU. Current projects are underway to: look at cancer stem cell treatment with CtBP inhibitors; develop and characterize CtBP-knockout cell lines for use as a model system; determine the oligomerization states of CtBP and analyze changes in the presence of inhibitors or substrate-domain mutations; and perform mouse studies to look at the role of CtBP2 knockdown in tumor ablation. Upcoming experiments will look at 4-Cl HIPP effects as anti-cancer therapy in mouse models. It is the hope that within the next few years, a CtBP inhibitor or combination treatment will be available for clinical trials. With continued progress in the effort to understand CtBP within a cancer context, results are promising.

Appendix 1: Simulation data comparing modes of inhibition

Using a predictive model of CtBP enzymatic activity in which addition of substrate and coenzyme involves an ordered mechanism, we present the following schematic, generated by Dr. William Royer:

Kinetic Simulations:

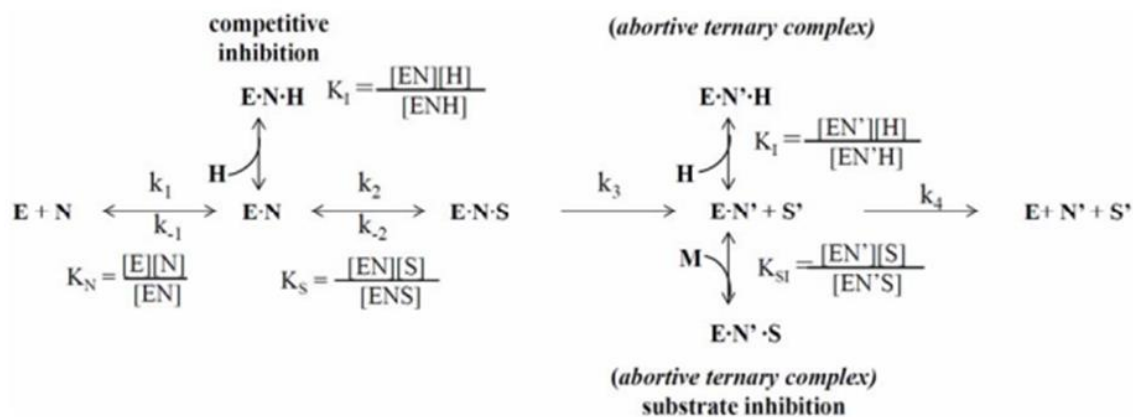
Shown below is the general scheme used to simulate the inhibition that could result through the formation of ternary abortive complexes upon binding either inhibitor or substrate to CtBP following the release of reduced product (S' –5-methylthio-2-hydroxybuterate [MTHB]) but not oxidized cofactor.

S = MTOB (substrate); S' = MTHB (reduced product)

N = NADH; N' = NAD^+

H = HIPP (inhibitor)

E = CtBP (enzyme)



From this scheme, the following equation for v_o was derived for HIPP inhibiting CtBP through formation of a ternary abortive complex:

$$v_o = \frac{V_{max} [N] [S]}{[S][N] + K_S[N] + K_N K_S + k_3 [N][S] \left(1 + \frac{[H]}{K_1} \right)}$$

In order to successfully derive simulation models, one must account for all forms in which the enzyme could potentially reside, leading up to the condition that one wants to test. For

example, for an abortive ternary complex, the enzyme can be unbound, bound to the coenzyme NADH, to coenzyme and substrate, to oxidized coenzyme NAD⁺, or finally bound to NAD⁺ and inhibitor. Since we had performed enzymatic experiments prior, these models can help to verify the data that we have seen. In fact, an abortive ternary complex simulates closely with the data that we saw from our K_i studies with HIPP (**Fig. A1. A-B**). The abortive ternary complex shows inhibition that effects V_{max} . Further, substituting [S] or substrate concentration, for [H], inhibitor concentration, one can use this same simulation to predict the effect of excess substrate on the reaction. This model (**Fig. A1. C**) shows an extremely similar pattern to the substrate inhibition that we observe once MTOB is found in excess of NADH (**Fig. A1. D**). The startling resemblance between this simulation should give weight to our hypothesis of HIPP as an inhibitor of ternary abortive complexes. Dr. Royer further simulated what data would look like if HIPP primarily interacted with free enzyme (**Fig. A1. E**). The pattern shows a change in K_m rather than V_{max} , suggesting that HIPP does not predominantly inhibit free enzyme. Nevertheless, as mentioned in the Discussion of Chapter 1, it is likely that HIPP can also bind competitively *in vivo*. Our assay favors a ternary abortive complex since we keep NADH in far excess, making access to free enzyme difficult. One way to potentially see the impact of HIPP binding free enzyme is by preincubating HIPP with monomeric CtBP protein and then performing an NADH disappearance assay. Efforts in the Grossman laboratory are being made to see inhibition caused by sequestering free enzyme.

Finally, simulations do not prove a hypothesis, but they can provide validity. There is some trial and error involved. In this particular case, it was necessary to approximate some variables. We are unsure of the rates of formation for many of the steps involved, and so these

numbers were estimated. However, together this work has helped us have a clearer picture of the mechanism of action of HIPP and its derivatives.

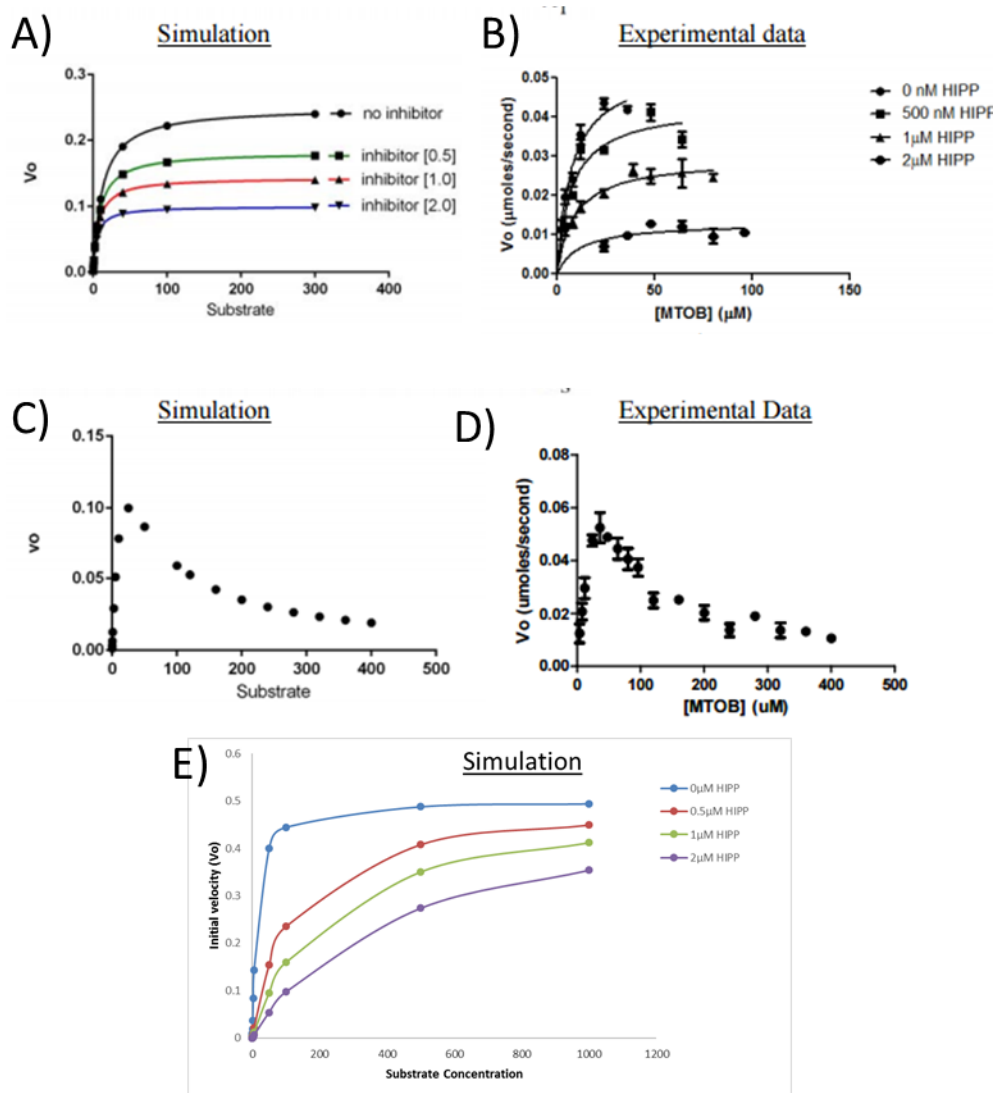


Figure A1_1: Simulated data using a model predictive of HIPP inhibition binding to an abortive ternary complex or empty enzyme. Models were simulated to explain the seemingly non-competitive binding of HIPP to CtBP. An abortive ternary complex **A)** shows the best fit to HIPP K_i data **B)**. This model also suggests substrate inhibition **C)** that mirrors what we see enzymatically with excess substrate **D)**. Another model was derived by Dr. William Royer to predict HIPP binding to free enzyme **E)**. It takes into account all states of the enzyme to the point of substrate binding. Total enzyme concentration, $[E]_t = [E] + [EH] + [EN] + [ENS]$. Deriving the equation to solve for $[ENS]$ we arrive at the equation $V_o = (V_{max}[N][S]) / ([N]K_s + K_n * K_s + (1 + [H]/K_i))$. Extending the substrate concentration, we can see that this model appears to differ by K_m rather than V_{max} , which less suitably fits what we see from our data.

Appendix 2: The importance of choosing the appropriate system for reproducible experimental data.

For the studies that we performed in Chapter 4, the majority of our effort was dedicated to creating and verifying a system that would reliably yield good results. In order to do so, the approach toward designing and carrying out experiments was changed multiple times. With the opportunity to be among the first to study CtBP in Dr. Grossman's lab at Virginia Commonwealth University, the design of these approaches allowed for numerous learning opportunities encompassing several skills, including cloning, assay optimization and troubleshooting. In addition, there are a number of lessons that were learned which can hopefully be applied toward future endeavors. The subsequent paragraphs discuss this learning process, and provide suggestions for creating an optimal system for studying CtBP based biology in cell-systems going forward.

The first attempts to study mutants in cancer cell systems involved creating a series of silent mutations within the protein coding sequence of CtBP2 in a pcDNA3.1 backbone (**Fig. A2, A**). The rationale for this approach claims that by creating silent mutations, one could then transfect exogenous CtBP2, treat cells with siRNA targeted to the normal sequence, and see downregulation of endogenous CtBP simultaneously with overexpression of exogenous CtBP. The system worked well (**Fig. A2, C**), but with both siRNA knockdown and overexpression, it was difficult to get reliable protein levels between independent experiments. Also, it was difficult to perform clonogenic or other long-term assays due to the transient nature of overexpression and knockdown. Attempts were then made to use lentiviral transduction to stably overexpress exogenous CtBP. Unfortunately, exogenous CtBP2 levels quickly dropped and endogenous expression was the predominant form synthesized (data not shown).

A third attempt utilized a new vector backbone, called pZ04, that was created by Dr. Priyadarshan Damle. It had a secondary SV40 promoter region that expressed a GFP, puromycin fusion gene in addition to silent backbone CtBP2. In this way, we hypothesized, it would be possible to stably transfect exogenous CtBP and select for those cells that integrated the plasmid into its genome by puromycin selection. The vector has the added benefit of expressing GFP, which allows a way to visually estimate transfection efficiency between different treatments under a fluorescence microscope. Fluorescence provides a means to bin cells based on high or low expressing GFP through FACS sorting. FACS sorting was attempted and cells expressing high GFP were FACS sorted and used for experiments. However, it was observed that expression was not standard between different cell lines. Inclusive with this observation was that expression level varied over passages, even while maintaining selective pressure. The result was an inability to determine if changes in phenotype were due to protein levels or the mutations themselves. An example is shown in **Figure A2,B-C** with migration data. Many of the trends are similar to that seen with the data presented in **Figure 4_2**, except for W324F. It was impossible from the assay alone to determine if the reduction in migration rate was due to lower levels of overall protein or because of an effect with W324F. These differences were not abrogated by siRNA knockdown of endogenous CtBP2.

After several months of attempting to resolve differences between stable cell lines, another attempt was made, using a CRISPR/cas 9 system to knockout CtBP2 in HCT116 p53^{-/-} cells. Frameshift mutations were appropriately introduced and screened, multiple cells were divided into single cell suspensions, and pure knockout lines were cultivated. This method has successfully knocked out CtBP2 (data not shown). The success of this effort was surprising, because CtBP2 continued-knockdown has been shown to cause significant amounts of cell death.

Work is ongoing to characterize these cells and their mechanism of proliferation. It has been anecdotally observed that they grow and proliferate at similar rates to normal HCT116 cell lines. Similar difficulties inherent with transfection efficiency with different mutants are seen, but overall the cells provide a cleaner background than previous systems. One concern for this cell line is that the survival mechanism is unknown, and as such, cells are probably more ‘non-normal’ than the comparison cell lines. Cells may have been able to find a way to circumnavigate CtBP regulation altogether and addition of exogenous sources may not be transcriptional utilized as normal.

With any system there are benefits and flaws. One of the consistent difficulties with studying CtBP is the apparent tight regulation of the protein in cells. In each system, overexpression normalizes with time, regardless of whether consistent selective pressure is applied. With more resources, a good next step could make use of an inducible promoter. A promoter containing a lentiviral shRNA doxycycline-inducible system has been implemented for CtBP and is being studied currently in the laboratory of Dr. Kevin Gardner at the NIH¹²⁰. A similar controllable expression system of CtBP would benefit our studies. A proposal for an alternative system would involve first creating CRISPR knock out cells for CtBP1. Since CtBP1 and CtBP2 are redundant, but CtBP2 is more imperative for nuclear localization, a large change in phenotype is not expected. Further, incorporating an shRNA inducible system could be coupled with CtBP2 expression in a vector containing silent mutations within the shRNA targeting region. The end result would be a chemically inducible system where all forms of CtBP are accounted for and under tightly regulated control that would hopefully limit variability between replicates, and provide a system for biological assessment of mutations and screening for CtBP-specificity with new drug therapies.

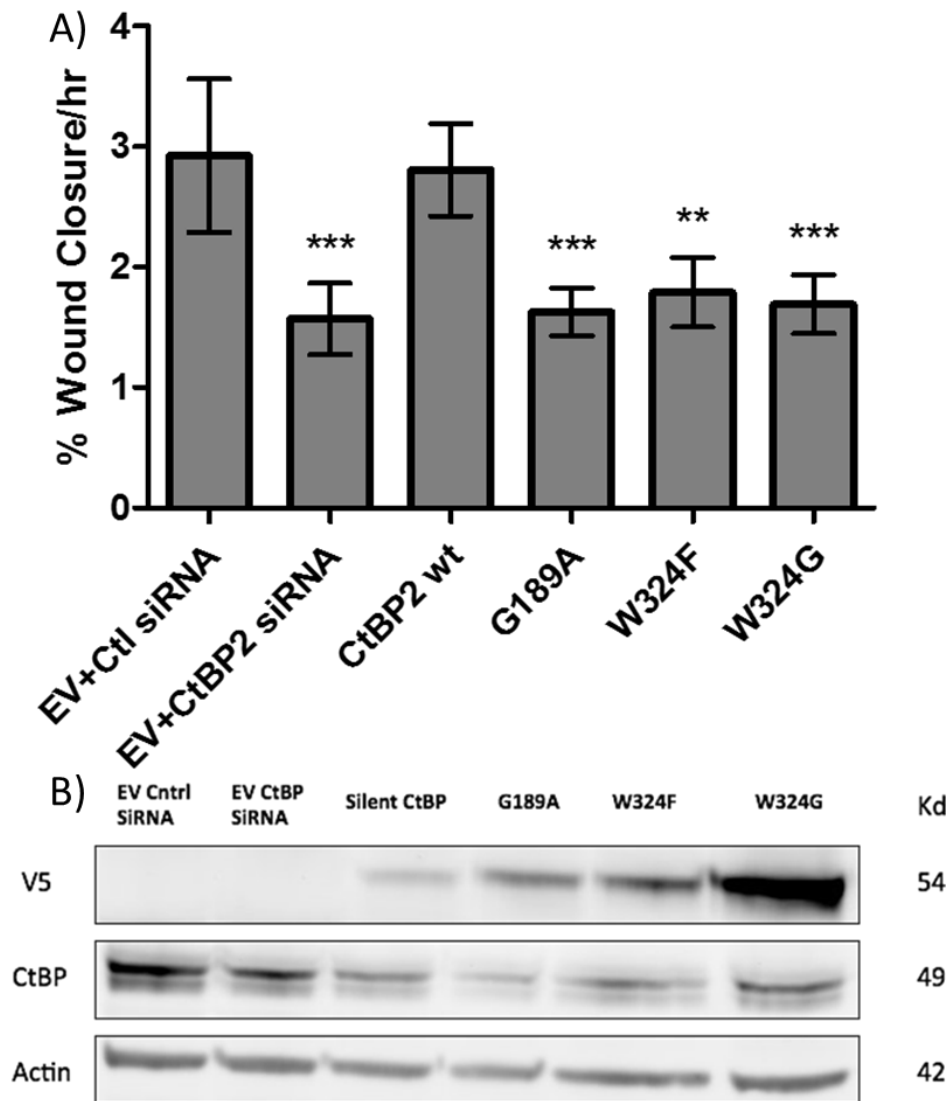


Figure A2_2. Stable transfection of CtBP2 mutants induces highly variable protein levels, confounding results. Stable CtBP2 mutants were generated in MDA-MB-231 cell lines. Endogenous CtBP2 was knocked down by siRNA, and a wound healing assay was performed, **A)**. Data represent $n \geq 3$ independent replicates with triplicate measures and error bars represent the 95% confidence interval. The corresponding Western Blot **B)** shows exogenous CtBP2 expression (V5), endogenous expression (CtBP), and an Actin loading control. **= p-value < 0.01, ***= p-value < 0.001 vs. control.

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Vita

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