A novel strategy to disrupt HDAC2 corepressor complexes to halt the cell division of cancer cells

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Abstract

The orderly packing of eukaryotic DNA depends on histones, which can be enzymatically modified to increase or decrease transcription. Histone-modifying enzymes affect gene expression by altering the level of compaction and the accessibility of DNA to binding proteins. While histone acetylation results in chromatin decondensation and increased transcription of the gene, histone deacetylases are associated with transcriptional repression and remove the acetyl groups from the histones which results in the condensation of chromatin. The deregulation of histone deacetylation has a role in several types of cancers. Histone deacetylase 2 (HDAC2) is recruited to promoters as a member of corepressor complexes such as Sin3 or NuRD which then repress transcription. Inhibition of HDAC activity can drive cultured tumor cells to growth arrest, differentiation and/or apoptic cell death. A novel strategy to dissociate these HDAC2 corepressor complexes was tested by fusing the TAT protein transduction domain with the C-terminal region of HDAC2. We were unable to purify the fusion protein using the IMPACT-CN protein purification system and test the hypothesis that the TAT-HDAC2 fusion protein will disrupt corepressor complexes negating their ability to repress target genes, in particular, p21 which regulates the cell cycle and induces apoptosis in cancer cells. We provide possible reasons and alternative mechanisms to purify the protein and determine the validating of our approach to disrupt HDAC2 corepressors.

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Introduction

1. Chromatin and Histones

Nucleosomes are the fundamental units of chromatin and are comprised of a histone protein octamer around which 146 base pairs of DNA are wound (Rao and others 2007; Roth and Allis 1996). The octamer contains two copies of the four core histones: H2A, H2B, H3 and H4 (Ocker and Schneider-Stock 2007; Rao and others 2007). Linker histones clamp entry and exit sites of DNA around the core histone octamer and, with other nuclear proteins, contribute to the formation of a condensed higher order chromatin structure (Rao and others 2007). Core and linker histones are the major regulators of chromatin condensation, thus their modifications regulate the structural stability of the chromatin fiber (Rao and others 2007).

The orderly packing of eukaryotic DNA depends on enzymatic modification of histones by the covalent addition of methyl, acetyl or phosphate groups to increase or decrease transcription (Karp 2005). These epigenetic changes (mitotically heritable alterations in gene expression that are not caused by changes in the DNA sequence) referred to as the "histone code" influence chromatin structure and thus regulate the accessibility of transcription factors to DNA (Ocker and Schneider-Stock 2007).

2. Histone Acetylation/Deacetylation

The acetylation and deacetylation of histones occurs at specific lysine (K) residues in the N-terminal tail of the four core histone proteins in the nucleus (Ocker and Schneider-Stock 2007; Roth and Allis 1996).

Histone acetylation results in chromatin decondensation and increased transcription of the gene (Karp 2005; Ropero and Esteller 2007). This is accomplished by

enzymes known as lysine acetyltransferases (KATs) (Fig 1). Histone acetylation has also been associated with other genome functions such as chromatin assembly, DNA repair, replication, and recombination (Ropero and Esteller 2007).

Eukaryotic cells also possess negative regulator mechanisms carried out by histone deacetylases (HDACs) which are involved in transcriptional repression and remove the acetyl groups from the histones resulting in the condensation of chromatin (Karp 2005) (Fig 1). The more compact chromatin structure represses gene transcription by limiting the accessibility of the transcription machinery (Ropero and Esteller 2007).

KAT and HDACs are present together at transcriptionally active genes (Sun, Chen, Davie 2007). At such sites in the genome these enzymes are mediating dynamic histone acetylation which is occurring at a rapid rate with acetyl groups being put on and taken off in minutes (Sun, Chen, Davie 2007). The reversible nature of acetylation of histone tails maintains a crucial balance between the activation and inactivation states of genes (Rao and others 2007).

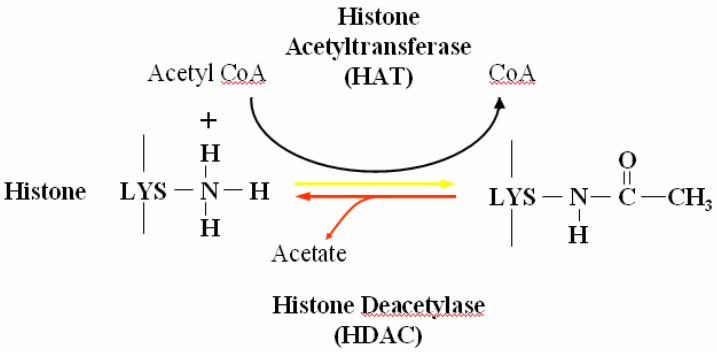


Fig 1: Acetylation and Deacetylation of Histones

3. Histone Deacetylases

There are four HDAC classes with several different HDACs in each class (Fouladi 2006). Class I HDACs are homologous to yeast Rpd3 and include HDACs 1, 2,

3 and 8. Class I HDACs are expressed ubiquitously in human cell lines and tissues and are found primarily in the nucleus (Ropero and Esteller 2007). Class II HDACs are homologous to yeast Hda1 and can be subdivided into two subclasses: IIa (HDAC 4, 7, and 9) and IIb (HDAC 6 and 10) (Ropero and Esteller 2007). Class II HDACs exhibit tissue-specific expressions and can shuttle between the nucleus and cytoplasm (Ropero and Esteller 2007). Class I and II HDACs share homology in their catalytic sites (Sekhavat, Sun, Davie 2007). Class III HDACs or sirtuins (SIRT1-7) include a group of proteins that are homologous with the yeast Sir2 family of proteins (Ropero and Esteller 2007). Class IV HDACs contain HDAC11 and are homologous with both class I and II (Ropero and Esteller 2007).

The role of HDACs is not restricted to their contribution to histone deacetylation, they also play a role in the deacetylation of non-histone proteins (Ropero and Esteller 2007). HDACs are key elements in the regulation of gene expression, differentiation and development, and the maintenance of cellular homeostasis.

4. HDAC2

HDAC2 is present in the nucleus of human cells (Sun, Chen, Davie 2007). It is involved in transcriptional repression regulated by the retinoblastoma protein Rb and is recruited to DNA through interaction with transcription factors and nuclear receptors (Ropero and Esteller 2007).

HDAC2 is also found in large multi-protein complexes called corepressor complexes that are recruited to promoters of genes by specific transcription factors to silence gene activity (Sun, Chen, Davie 2007). HDAC2 is one of the histone-modifying enzymes that regulates gene expression by remodeling chromatin structure and is present

in the Sin3A and nucleosome remodeling and deacetylase (NuRD) corepressor complexes (Sun, Chen, Davie 2007).

5. Phosphorylation of HDAC2

Phosphorylation is a post-translational modification which enhances the enzymatic activity of HDAC1 and 2 (Sun, Chen, Davie 2007). HDAC2 is tri-phosphorylated in MCF-7 cells at Ser^{394}, Ser^{422}, and Ser^{424} by the protein kinase CK2. Phosphorylation of HDAC2 seems to provide the switch in directing the complex composition and chromatin location (Sun, Chen, Davie 2007). Unmodified HDAC2 is associated with the coding regions of transcribed genes, while phosphorylated HDAC2 is located at promoters (Sun, Chen, Davie 2007). pHDAC2 promotes enzymatic activity, regulates complex formation and has an effect on transcriptional repression.

6. Rb protein and RbAp48

The tumor-suppressor gene Rb requires the recruitment of class I HDACs to repress gene transcription. Thus, the loss of class I HDAC activity could induce the expression of genes regulated by Rb, thereby suppressing their protective role in tumor development (Ropero and Esteller 2007; Roth and Allis 1996).

The Rb-binding protein, RbAp48 is a member of an evolutionary conserved subfamily of WD-repeat proteins (Roth and Allis 1996; Yarden and Brody 1999). RbAp48 is homologous to RbAp46 and both were first isolated based on the interaction with the carboxyl terminus of the Rb protein (Nicolas and others 2000; Yarden and Brody 1999). RbAp48 interacts with histone H4 and is often seen as a molecular platform that allows for the formation of various different multimolecular complexes (Nicolas and

others 2000). It is found in ATP-dependent remodeling complexes and plays a role in their sequential formation (Nicolas and others 2000). RbAp48 is a core component of both the Sin3A and NuRD corepressor complexes.

RbAp48 and 46 are components of at least three other distinct nucleosome-modifying complexes: Drosophila nucleosome-remodeling factor NURF, chromatin assembly factor 1 (CAF-1) and Hat1 (type B (cytoplasmic) histone acetylase involved in chromatin assembly) (Zhang, Vo, Goodman 2000). Type B acetyltransferases and CAF-1 are involved in chromatin assembly (Zhang, Vo, Goodman 2000). RbAp48 is also associated with the coactivator CREB binding protein (CBP) (Zhang, Vo, Goodman 2000). CBP and p300 are KATs that have important roles in gene regulation and have been implicated in a variety of cellular processes including regulation of the cell cycle, differentiation, DNA repair and apoptosis (Zhang, Vo, Goodman 2000). CBP and its homologue p300 are prototypical transcriptional coactivators (Zhang, Vo, Goodman 2000).

7. HDAC1 and 2 Corepressor Complexes

KATs and HDACs work together with transcriptional co-activators and repressors and other DNA-modifying enzymes in large multi-protein complexes regulating gene transcription in eukaryotic cells (Ocker and Schneider-Stock 2007).

HDAC1 and HDAC2 are found in the NuRD and Sin3A multi-protein complexes which are recruited to promoters by DNA binding proteins (Sun, Chen, Davie 2007; You and others 2001). The NuRD complex contains HDAC1 and 2 and ATP-dependent chromatin remodeling protein CHD4 (Mi-2) and MTA2 (You and others 2001). NuRD functions in transcriptional repression but may also have a role in remodeling,

recombination, replication and DNA repair (Sekhavat, Sun, Davie 2007). The Sin3A complex is recruited by DNA-binding transcription factors such as unliganded nuclear hormone receptors and Mad/Max heterodimer to promoters (You and others 2001).

The phosphorylation of HDAC2 is required for its binding to RbAp48 which in turn forms a platform for other proteins to form the Sin3A and NuRD complexes which are then recruited by transcription factors p53, Rb, YY1, NF-κB, Sp1 and Sp3 to specific promoters (Sun, Chen, Davie 2007).

8. p21 and p53

The p21 protein has a role in cell cycle control and during DNA damage response. It is a tumor suppressor gene and the best example for showing that proliferation and apoptosis in cells are tightly coupled (Ocker and Schneider-Stock 2007). p21 negatively modulates cell cycle progression by inhibiting the activity of cyclin/cdk2 complexes which drive the cell through the G1 checkpoint. p21-dependent cell cycle arrest is one of the mechanisms by which p21 can prevent cells from apoptosis because it allows for repair and prevents DNA damage (Ocker and Schneider-Stock 2007). p21 also inhibits DNA replication through its ability to bind the proliferating cell nuclear antigen (PCNA) which is required for both replicative DNA-synthesis and DNA-repair (Ocker and Schneider-Stock 2007). Cells without p53/p21 cannot maintain cell cycle arrest and begin catastrophic nuclear division that leads finally to cell death (Ocker and Schneider-Stock 2007).

Normal p53 is a short lived protein that is maintained at low levels (Zhou and others 2000). However, in response to cell stress or DNA damage, p53 is stabilized and accumulates in the nucleus where it functions as a transcription factor inducing p21, G1

cell cycle arrest, and apoptosis (Zhou and others 2000). p53 is phosphorylated and acetylated under stress conditions. The acetylation of p53 serves to promote protein stability and activation, inducing checkpoints in the cell-division cycle, permanent cell-division arrest and cell death (Ropero and Esteller 2007).

In response to DNA damage, p53 protein binds directly to the p21 promoter at Sp1 site, a region where p53 and HDAC1 compete for binding (Ocker and Schneider-Stock 2007). Dysregulation of p21 expression and HDAC function are commonly observed in a variety of human tumor diseases (Ocker and Schneider-Stock 2007). Both mechanisms are closely interconnected and lead in a synergistic manner to a growth advantage for the tumor cell (Ocker and Schneider-Stock 2007).

1) Cell Cycle Control

The deregulation of histone acetylation has a role in several types of cancers (Fouladi 2006). Thus, HDACs are associated with the development of cancer (Fouladi 2006). HDACs are involved in the deacetylation of chromatin proteins and non-histone proteins which regulate important functions that in turn regulate cellular homeostasis such as cell-cycle progression, differentiation and apoptosis (Ropero and Esteller 2007). In the poised state (i.e. ready for transcription but not transcribed) the p21 promoter has a higher load of HDAC versus KAT activity and hence is in a chromatin state that does not support transcription. The p21 protein inhibits cyclin-dependent kinase 2 (cdk2) which drives the cell through the G1 checkpoint. However, if the expression of p21 is activated by a damaged cell, then the cell cycle is arrested (Karp 2005).

9. Cancer

Cancer is the result of a wide variety of genetic and genomic alterations (amplifications, translocations, deletions, and point mutations) (Ropero and Esteller 2007). These alterations tend to result in the activation of oncogenes and the inactivation of tumor-suppressor genes (Ropero and Esteller 2007). However, cancer is not solely restricted to genetic changes, but epigenetic changes as well (Ropero and Esteller 2007). A typical characteristic of human cancer is the deregulation of DNA methylation and post-translational histone modifications. In particular, histone acetylation has the fatal consequence of gene transcription-deregulation (Ropero and Esteller 2007). Data gathered from studies of normal tissues, primary tumors, and human cancer cell lines indicate that the loss of acetylated Lys16 (K16-H4) and trimethylated Lys20 (K20-H4) of histone H4 is a common event in human cancer (Ropero and Esteller 2007). These changes have been shown to occur early in tumorogenesis, which indicate that the global loss of monoacetylated and trimethylated forms of histone H4 is a crucial event in cancer development (Ropero and Esteller 2007).

The abnormal targeting or retention of HDACs to DNA regulatory regions is observed in many cancers (Sekhavat, Sun, Davie 2007). A number of studies show an altered expression of individual HDACs in tumor samples. An increased expression of HDAC 1 is observed in gastric, prostate, colon, and breast carcinomas (Ropero and Esteller 2007). The over expression of HDAC2 has been found in cervical and gastric cancers (Ropero and Esteller 2007). These findings suggest that transcriptional repression and the abnormal recruitment of HDACs to their promoter regions could be a common phenomenon in tumor onset and progression (Ropero and Esteller 2007). A typical

example of this transcriptional repression is the cyclin-dependent kinase inhibitor p21 which inhibits cell-cycle progression and whose expression is lost in many different tumors (Ropero and Esteller 2007).

10. HDAC inhibitors

Cancer cells express an abnormal amount of HDACs and/or express mutant proteins that mis-target HDACs. Knowing this, there is an interest in finding pharmacological agents that inhibit HDAC activity and as a result, inhibit transcriptional repression of the p21 gene (Fouladi 2006).

Treatment of cells with HDAC inhibitors (HDACi) results in the re-expression of repressed genes leading to cellular differentiation, inhibition of cell cycle progression and induction of apoptosis (Fouladi 2006). Many of the genes affected by HDAC inhibitors are key regulators of crucial biological processes in tumor progression (Ropero and Esteller 2007). G1 cell cycle arrest and apoptosis are characteristic of HDAC inhibitors which results in the internucleosomal breakdown of chromatin, the down regulation of cyclin D1 and the up regulation of p21 (Fouladi 2006; Zhou and others 2000). Furthermore, HDACi affect the rapid turnover of acetylation of histones associated with active and competitive genes (Sekhavat, Sun, Davie 2007). The deregulation of histone acetylation is thought to play a role in the pathogenesis of hematological and solid tumors by changing the chromatin structure and transcription of genes involved in cell cycle control, differentiation or apoptosis (Fouladi 2006).

p21 is one of the most commonly induced genes by HDACi and is correlated with an increase in the acetylation of histones in the promoter region (Marks and others 2004). Following the exposure to class I and II HDAC inhibitors, the acetylation state of

several proteins is increased following induction of p21, in particular: the p53, α-tubulin, Hsp90, MyoD and GATA-1 proteins (Marks and others 2004). In addition, cytoplasmic lipid droplet accumulation is seen in human breast cancer cell lines when cells begin to differentiate after treatment with HDACi (Zhou and others 2000). Some anti-cancer affects for HDACi include inhibition of angiogenesis and invasion and immunomodulatory effects (Ocker and Schneider-Stock 2007).

Defining downstream effects of HDAC inhibition could provide a mechanistic rationale for the use of combination therapies with HDAC inhibitors (Marks and others 2004). Although promising, HDAC inhibitor technology is not 100% effective. $HDAC_i$ only alter the expression of less than 10% of expressed genes and are based on inhibiting a broad spectrum of HDACs (Marks, Miller, Richon 2003).

1) HDACi and Clinical Trials

Recently, suberoylanilide hydroxamic acid (SAHA) was approved by the Food and Drug Administration (FDA) for the treatment of cutaneous T-cell lymphomas and is the only HDACi currently in clinical use. SAHA was found to be an inhibitor (at nanomolar concentrations) of partially purified HDAC and induces p21 expression (Fouladi 2006; Marks, Miller, Richon 2003). However, SAHA is toxic when administered to adults; side-effects include anorexia, dehydration, diarrhea, nausea and fatigue (Fouladi 2006). As a result, the desire to find an HDACi to a specific class of HDACs without inducing toxic side-effects is highly sought after.

2) Trichostatin A

Class I and II HDACs are inhibited by trichostatin A (TSA) (Fig 2), SAHA and related compounds (Marks, Miller, Richon 2003). TSA was the first natural product

hydroxamate that inhibits HDACs directly (Marks, Miller, Richon 2003). It is a potent and specific inhibitor of HDAC (Ailenberg and Silverman 2003). TSA modulates transcriptional activity thereby affecting a broad variety of cellular processes such as cell cycling, differentiation, and apoptosis, culminating in the inhibition of cell proliferations, angiogenesis, and metastasis (Ailenberg and Silverman 2003). TSA results in the enhanced acetylation of core histones thereby disrupting chromatin organization within living cells (Rao and others 2007).

A study done by Sekhavat et al. found that TSA is a competitive inhibitor of HDAC2 but does not affect the extent of interaction between HDAC1 and HDAC2 (Sekhavat, Sun, Davie 2007). Furthermore, TSA does not significantly alter the association of HDAC1 and 2 with the Sin3A complex (Sekhavat, Sun, Davie 2007).

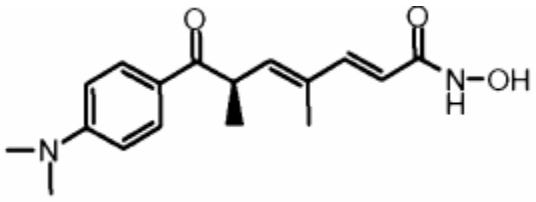


Fig 2: Chemical structure of TSA

11. Emerging Anti-Cancer Therapies

An increasing challenge in cancer treatment is to target selectively and distinguish between the tumor and normal cells (Ailenberg and Silverman 2003). A main problem in chemotherapy is the genetic instability of cancer cells that renders them non-sensitive to chemotherapy (Ailenberg and Silverman 2003).

A major goal of anticancer therapy is to specifically kill tumor cells while leaving normal cells unharmed (Wadia and Dowdy 2005). Traditional cancer therapies such as

chemotherapy are often poorly specific for cancer cells since only certain proteins and/or protein-protein interactions are specifically altered or deregulated in cancer cells (Wadia and Dowdy 2005). As a result, an attractive strategy for anti-cancer therapy has been to develop molecules that target and modulate tumor-specific proteins and protein-protein interactions (Wadia and Dowdy 2005).

One mechanism to modulate the biology of cancers involves the direct introduction of peptides, full-length proteins and/or protein functional domains into tumor cells (Wadia and Dowdy 2005). The ability to manipulate cell biology at the protein level, without the use of DNA based methods would provide a powerful tool for understanding and affecting complex biological processes and thus, for the treatment of a variety of human diseases including cancer (Wadia and Dowdy 2005).

12. Cell penetrating peptides

The direct intracellular delivery of proteins is difficult to achieve due to the plasma membrane barrier, which prevents uptake of macromolecules (Wadia and Dowdy 2005). Classical manipulation methods of mammalian cells have been achieved by the transfection of expression vectors, microinjection or diffusion of peptidyl mimetics (Becker-Hapak, McAllister, Dowdy 2001). Although successful, these methods are laborious, not easily regulated and often have poor specificity, unwanted side-effects or are toxic. Cell penetrating peptides (CPP) are short cationic peptide sequences that get around this problem (Jones and others 2005).

A protein that can cross cell membranes and transactivate a viral genome was first described in 1988 by Green and Loewenstein and Frankel and Pabo (Becker-Hapak, McAllister, Dowdy 2001). Other transduction domains have also since been described

such as Antennapedia (Antp) from Drosophila and HSV VP22 from the herpes simplex virus (HSV). The exact mechanism of protein transduction across cellular membranes remains unclear in any of the domains studied (Becker-Hapak, McAllister, Dowdy 2001). The transduction of these cell-mediating peptides does not appear to be affected by cell type and can efficiently transduce into almost 100% of cells both in vitro and in vivo with no apparent toxicity (Wadia and Dowdy 2005). Protein transduction domains have also been used to induce the intracellular uptake of DNA, antisense oligonucleotides and small molecule drugs (Wadia and Dowdy 2005).

The amino-acid sequences of protein transduction domains for differing peptides are as follows: Antennapedia (RQIKIWFQNRRMKWKK), Transportan (AGYLLGKINLKALAALAKKIL), and Polyarginine (RRRRRRRRRR) (Jones and others, 2005).

2) <u>TAT Transduction Domain</u>

The human immuno virus-1 transactivator of transcription protein transduction domain (HIV-1 TAT-PTD) CPP can easily cross through the plasma and cell membrane. The HIV-1 TAT protein is an essential viral regulatory factor involved in the transactivation of genes involved in the HIV long terminal repeat (Wadia and Dowdy 2005). TAT plays a critical role in viral replication as a transcriptional activator (Wadia and Dowdy 2005). The full length of the TAT protein transduction domain is 86-amino-acid although as little as residues 47-57 (YGRKKRRQRRR) are needed for the protein to work efficiently (Wadia and Dowdy 2005).

The TAT protein transduction domain can modulate the biology of living organisms and be used to treat diseases such as cancer by the direct cellular delivery of

proteins and peptides (Wadia and Dowdy 2005). (Becker-Hapak, McAllister, Dowdy 2001) has developed a protocol for fusing the Tat-ptd to a desired protein and amplification procedures in order to effectively transduce a protein into a cell.

Research Hypothesis and Aims

My hypothesis was that the C-terminal region of HDAC2 (residues 323-488) would be phosphorylated *in situ* by protein kinase CK2 and bind to RbAp48 thereby sequestering RbAp48 leading to the disruption of the Sin3A and NuRD HDAC1/2 corepressor complexes negating their ability to repress target genes, in particular p21.

The aims of my project were to develop a TAT-HDAC2-C-terminal fusion protein that would be transduced into MCF-7 breast cancer cells and test for the disruption of the Sin3A and NuRD complexes as well as growth inhibition of the cells.

Materials and Methods

1. Preparation of fusion protein

Residues 47-57 of the TAT-ptd were fused with the C-terminal residues (323-488) of HDAC2 by Jenny Yu in Dr. Jim Davie's lab at the Manitoba Institute of Cell Biology to yield the fusion protein TAT-HDAC2-C-terminal. The protein was expressed, amplified and purified using the IMPACT-CN protein purification system from New England Biolabs using an Intein tag in the pTYB1 vector.

2. DNA Ligation Procedures

The TAT-HDAC2-C-terminal fusion protein was inserted into the pTYB1 vector (New England Biolabs) using DNA ligation kits from New England Biolabs. The following quantities of solutions were added to an Eppendrof test tube: 1 μL of 5x ligation buffer, 0.5 μL of T_4, 1 μL of the pTYB1 vector, 2 μL of the TAT-HDAC2-C-terminal and 0.5 μL of H_2O. Tubes were centrifuged for 1 min at 6000 rpm (Microlite Thermo IEC) and then placed in 14°C for 1 hour to allow the insertion of the fusion protein sequence into the pTYB1 vector.

3. Construct Sequencing

The pTYB1-TAT-HDAC2-C-terminal construct was transformed into the *Escherichia coli* strain BL21 bacteria (Invitrogen) and purified using the DNA Purification from Cells in Media Procedures described below. The pTYB1 vector contains the T7 Universal forward Primer and Intein Reverse Primer for sequencing initiation. Constructs were sent for sequencing to Roberts Research Institute in London, Ontario. Results were received electronically via email.

4. Competent Cell Preparation Procedure

One mL of *E. coli* BL21 bacteria was transferred to a sterile 100-mL flask containing 50 mL of Luria-Bertani (LB) broth (10g Tryptone, 5g yeast extract, 10g NaCl per 1 L and pH was adjusted to 7.0 with NaOH) without antibiotics. Cells were incubated cells at 37°C with shaking at 220 rpm (New Brunswick Scientific C25 Incubator Shaker) until OD_{600} reached 0.5 (Pharmacia Biotech Ultrospec 3000). Once the culture reached the appropriate OD, it was chilled on ice for 20 minutes following which cells were collected by centrifugation (Sorvall RC5-C *Plus*, SS34 fixed rotor) at 3000 rpm for 15 minutes at 4°C in four centrifuge tubes each containing 10 mLs of sample. Cells were resuspended in 5 mL of TSS buffer (for 50 mL: 5g Polyethylene Glycol (PEG), 0.30 g $MgCl_2*6H_2O$, 2.5 mL DMSO, up to 50 mL LB broth) and then aliquoted 50µL into 10 1.5 mL microcentrifuge tubes and stored in -70°C freezer. *E. coli* strain DH5α (Invitrogen) competent cells were made following the same procedure.

5. BL21 bacterial transformation

100 ng of pTYB1-TAT-HDAC2-C-terminal was added to 50 µL of BL21 in each 1.5 mL Eppendorf microcentrifuge tube. Test tubes were placed on ice for 30 minutes and then heat shocked at 37°C for 40-60 seconds. Test tubes were replaced on ice for 2 minutes after which 80-100 µL of LB media was added to each tube. The culture was placed in the shaker for 1 hour and then were plated on Kanamycin-LB plates and placed in 37°C overnight to allow bacterial colony growth.

Kanamycin (Kana) or Ampicillin (AMP)-LB plates: 100 µL of the desired antibiotic was added to 1 L of autoclaved LB medium (LB medium, 15g/L agar) and

mixed gently. Medium was poured into plates (FisherBrand, 100 x 15mm) while warm and allowed to solidify. Plates were stored at 4°C.

6. DNA Purification from Cells in Media Procedures

After transformations, cell colonies of BL21 and *E. coli* DH5α were picked up from LB plates the next morning and transferred into 4 mL of LB media and shook over night at 37°C. The next morning the test tubes were centrifuged at 7000 rpm for 10 minutes (RC5-C SS-34 rotor). The liquid was discarded and the pTYB1-TAT-HDAC2-C-terminal construct was purified from the BL21 pellet using the plasmid purification buffers supplied from QIAGEN with the following steps: 250 μL of P1 buffer with RNAse A and 250 μL of P2 buffer were added to the BL21 pellet. After 5 minutes 350 μL N3 buffer were added to pellet. The total contents of test tubes were mixed gently. The mixture was then centrifuged at 13,000 rpm for 15 minutes (MicroLite Thermo IEC). The supernatant was transferred to column test tubes (QIAquick Spin Columns, QIAGEN) and then centrifuged again for 30-40 seconds at 6000 rpm (MicroLite Thermo IEC) following which the supernatant was discarded. 500 μL of PB binding buffer were added to the column test tubes, centrifuged for 40 seconds at 6000 rpm and then discarded. Next, the column test tubes were washed with 750 μL with PE Wash Buffer and centrifuged for 30-40 seconds at 6000 rpm. PE Wash Buffer contains alcohol which could affect the construct. To ensure complete removal of the PE Wash buffer from the column and construct, the test tubes were centrifuged again for 2 minutes and liquid contents were discarded. The quick spin columns were transferred to new 1.5 mL Eppendrof tubes. To release the DNA contents from the columns, 25 μL of Elution Buffer was added to the

columns for 1 minute before centrifuging for 2 minutes at 6000 rpm twice. The desired DNA plasmid pTYB1-TAT-HDAC2-C-terminal was in the flow-through.

7. Plasmid cutting procedures

To determine if the TAT-HDAC2-C-terminal had successfully been inserted into the vector, the plasmids were digested in order to determine if the size insert was correct. 1μL of 2x buffer, digestion enzyme Nde 2 (New England BioLabs) and restriction enzyme Xho1 (New England BioLabs) were added into a 1.5 mL tube with 4 μL of pTYB1-TAT-HDAC2-C-terminal to each tube; 3 μL of H_2O were added for a total of 10 μL. Contents were mixed for a second and then placed in 37°C H_2O overnight.

8. 1% Agarose Gel Making and Running Procedures

100 g of agarose (Invitrogen) was mixed with 100 mLs of TAE buffer (40 mM Tris-acetate, 1mM EDTA, pH 8,3). The mixture was heated in the microwave to boiling point and then kept warm over a hot plate while 10 μL of ethidium bromide was added to the mixture. The mixture was then poured into agarose gel plates (FisherBrand) and solidified. Gels were run at 150 V for 1 hour with 20 μL sample of the digested pTYB1-TAT-HDAC2-C-terminal construct.

9. DNA Gel Purification Procedures

The pTYB1-TAT-HDAC2-C-terminal band was cut out of the Agarose gel and put it in capped column test tubes with filters (QIAquick Spin Columns, QIAGEN). The DNA was purified from the gel using the QIAEX II Gel Extraction kit from QIAGEN and stored at room temperature with the following procedures: 1 g of the gel with the insert was cut and placed in a capped column test tubes with filters (QIAquick Spin Columns, QIAGEN). 3 μL of QG buffer were added to the test tubes which were placed tubes in

65°C for half an hour. Next, the tubes were centrifuged at 10,000 rpm in (MicroLite Thermo IEC) for one minute and the liquid was discarded. Following which, 300 µL of QG buffer was added to the tubes to clean the gel. After centrifuging for 1 minute, the liquid was discarded and 750 µL of wash buffer was added to the column tubes. Tubes were centrifuged for 3 minutes and the flow-through was discarded. The quick spin columns were transferred to new 1.5 mL Eppendrof tubes. To release the DNA contents from the columns, 25 µL of Elution Buffer was added to the columns for 1 minute before centrifuging for 2 minutes at 6000 rpm twice. The desired DNA plasmid pTYB1-TAT-HDAC2-C-terminal was in the flow-through.

10. Protein Purification and Chitin-column Chromatography

Protein purification was completed following the IMPACT-CN Protein Purification System from New England BioLabs. See Appendix A.

I. Culture Preparation:

Transformed BL21 with pTYB1-Tat-HDAC$_2$ were inoculated in 1 L of LB media or Terrific Broth (TB) (YTG base: 12 g Tryptone, 24 g Yeast Extract, 4 mL glycerol in 900 ml H_2O and autoclaved. Potassium Phosphate solution: 2.31 g KH_2PO_4 monobasic, 12.54 g K_2HPO_4 dibasic in 100 mL in H_2O. Solutions autoclaved separately and then mixed) with 100 µg/mL ampicillin and incubated in a 37°C shaker (New Brunswick Scientific C25 Incubator Shaker).

II. Induction and Cell Harvest:

Once the culture reached an OD_{600} of 0.5-0.6 it was induced with 0.3 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 3 hours at 37°C in an air shaker.

Following induction, the culture was centrifuged at 4000 rpm for 20 min at 4°C (RC5-C, GSA rotor) and supernatant was discarded. Unused bacterial pellets were stored in -70°C.

III. Crude Cell Extracts:

BL21 bacterial pellets were resuspended in 10 mL of Cell Lysis Buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 1 mM EDTA, Complete mini-EDTA-free plus protease inhibitor cocktail tablets) and sonicated 10x for 30 sec each (Sonifer cell disruptor). Clarified extracts were obtained by centrifugation at 10,000 rpm for 40 min at 4°C (RC5-C, SS34 rotor). Supernatant (clarified extract) was saved and pellets discarded.

The following steps were completed at 4°C:

IV. Chitin Column Equilibriation:

Six mL of chitin bead slurry (New England Biolabs) was aliquoted into a 20 mL column (BioRad Disposable Chromatography Columns). Columns were equilibrated with 10x the chitin bead volume with column buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 1 mM EDTA).

V. Loading, Wash, On-column Cleavage Reaction and Target Protein Elution:

Following equilibration, the clarified extract was slowly loaded onto the chitin columns. Columns were then washed with 4x the clarified extract volume with column buffer. On-column cleavage reaction was achieved by flushing the column quickly with 3 column volumes of Cleavage Buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 1 mM EDTA, 50 mM DTT) to evenly distribute buffer throughout the column and then capped and left at 4°C overnight. The target protein was eluted from column by using column buffer the following morning. One mL fractions were collected for three column volumes.

11. Gel Electrophoresis and Western Blot Transfer

Western blot analysis was run on samples from the crude cell extracts and from each step of the chitin column. 15 μL samples were diluted in 5 μL 6x SDS sample buffer, separated by 10% (SDS-PAGE) and transferred onto a nitrocellulose membrane via electro-transfer. The gel was analyzed by immunoblotting with antibodies specific to the C-terminal residues of polyclonal HDAC2 (Affinity Bioreagents) and the chitin-binding domain located in the intein tag of the $pTYB_1$ vector (New England Biolabs).

12. Total Histone Deacetylase Preparation

Human Embryonic Kidney 293 cells and MCF-7 breast cancer cells were harvested and lysed in NP-40 buffer (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1.5 mM $MgCl_2$, 0.65% NP-40) containing 1mM phenylmethanesulphonylfluoride (PMSF) and protease inhibitors (Complete Mini EDTA-free cocktail tablets, Roche Diagnostics). The cells were sonicated six times for 15 seconds each at 40% output (Sonifer cell disrupter). The supernatant (cell lysate) was collected and discarded by centrifugation at 13 000 rpm for 10 min (MicroLite Thermo IEC). The cell pellet was resuspended in 200 μL 2x sample buffer (6x sample buffer: 1.5 ml 1M Tris ph 6.8, 3 ml glycerol, 60mg Bromophenol Blue, 1.2g SDS, 1.2ml β-mercaptoethanol, ddH_2O up to 10 ml total) and stored at -20°C.

13. Cell Culture

Human breast cancer cell lines MCF-7 (T5) [estrogen-receptor (ER) positive and estrogen dependent] were cultured in Dulbecco's modified Eagle's medium (DMEM) from GIBCO supplemented with 5% fetal bovine serum (GIBCO), antibiotic-antimycotic

(GIBCO), 200 mM L-glutamine (GIBCO), 1% Penicillin-streptomysin, and 3% glucose. Cells were cultured in 37°C humidified incubator with 5% CO_2.

14. Cell treatment with TSA

MCF-7 cells (1.0×10^5) were plated into 7 sterile tissue culture Petri dishes (Cell Culture dish 100mm x 20mm, Corning Inc.). After 4 days the cells became 75-85% confluent. Each plate was incubated with 500ng TSA. Control plates were incubated with 5 µL of absolute ethanol. Cell morphology and growth was monitored over a 72h time period at five intervals: 0.5h, 6h, 12h, 24h, 48h and 72h. Pictures of cell cultures were taken with an Olympus Fe-115 digital camera.

Results

Verification of pTYB1-TAT-HDAC2-C-terminal constructs.

To determine if the construct was correct before continuing on to further steps, the construct was purified and sent to Roberts Research Institute DNA Sequencing Facility in London, Ontario for sequencing. The construct was verified to be correct as all of the necessary sites were identified in the sequence as seen in Fig 3. The Nde 1 translation initiation codon, Xho1, Sap 1 restriction site, intein tag, intein cleavage site and Intein Reverse Sequencing Primer were verified from page 8 of the Impact-CN Protein Purification Manual (New England Biolabs). Residues 47-57 of TAT were confirmed by Wadia, 2005. HDAC2 sequence was confirmed from by BLAST sequencing from National Center for Biotechnology Information (NCBI).

Fig 3: pTYB1-TAT-HDAC2-c-terminal construct sequence.

The construct was sequenced by Roberts Research Institute DNA Sequencing Facility in London, Ontario, Canada. Coloured boxes delineate notable sections of the construct. Yellow: Nde 1 translation initiation codon. Blue: Residues 47-57 of TAT-ptd. Red: HDAC2. Lime Green: HDAC2 Nuclear Localization Signal. Orange: Xho1. Purple: SAP1 Restriction Site. Pink: Intein Tag with Chitin-binding Domain. Black: Stop Codon. ▲ Indicates intein cleavage site

Size of pTYB1-TAT-HDAC2-C-terminal.

To accurately assess the success of the protein purification system, the size of pTYB1-TAT-HDAC2-C-terminal was determined to be 65 kDa. The TAT-ptd has a size of 1 kDa, The HDAC-C-terminal region (323-488) used in the fusion protein is 13 kDa, and the Intein Tag = 55 kDa. Consequently, the molecular mass of the intact HDAC2 enzyme is 69 kDa which is very close to the molecular mass of the full fusion protein construct.

Determination of pTYB1-TAT-HDAC2-C-terminal in crude lysate.

BL21 bacteria was transformed with the pTYB1-TAT-HDAC2-C-terminal construct in one liter of LB broth at 37°C and then induced with 0.5 mM IPTG for four hours. Following induction, the bacteria were pelleted and a crude lysate prepared as described in Material and Methods. MCF-7 breast cancer and Human Embryonic Kidney cell nuclei (HEK293) were used as controls for the construct. HDAC2 is detected in both MCF-7 breast cancer and HEK293 cell lysates as well as in the crude lysate made from the BL21 bacteria (Fig 4). Given that the sizes of the fusion protein and full length HDAC2 are very similar, distinguishing between the construct and full-length HDAC2 can be problematic. However, HDAC2 is found only in mammals and is not naturally synthesized in *E. coli* bacteria. Thus, it is fairly certain that the HDAC2 detected in the crude lysate from BL21 is from the pTYB1-TAT-HDAC2-C-terminal construct since any other form of HDAC2 would not be present in the bacteria cells. Furthermore, the BL21 lane shows HDAC2 detected at 14 kDa, which is the size of the TAT-HDAC2-C-terminal protein size (Fig 4). According to the IMPACT-CN system, *in vivo* protein cleavage can occur if the temperature of induction is too high. Fig 4 shows that some autoproteolysis

of proteins may have occurred in the bacteria resulting in cleavage between the Intein tag and the TAT-HDAC2-C-terminal insert. In addition, the chitin beads were tested following the chitin column elution via a Coomassie blue test. Protein was not detected on the beads following elution or in the eluted fractions. These results indicate that the pTYB1-TAT-HDAC2-C-terminal construct is not present on the chitin resin before or after the on-column cleavage reaction with DTT.

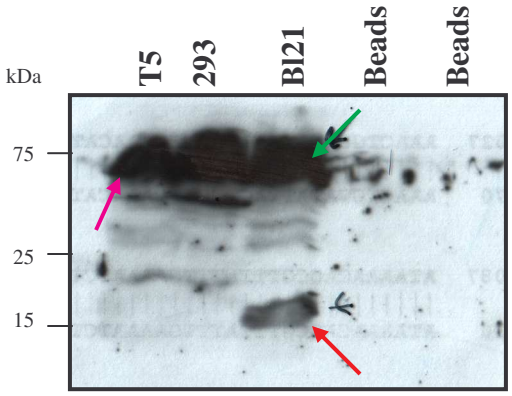


Fig 4: pTYB1-TAT-HDAC2-C-terminal construct present in crude lysate from BL21.

MCF-7 (T5) and HEK293 nuclei were used as controls for HDAC2. BL21 bacteria transformed with the pTYB1-TAT-HDAC2-C-terminal construct was grown in 2 liters of LB media in at 37°C air shaker until the culture reached OD_{600} 0.493. The culture was induced with 0.5 mM IPTG for 4 hours at 37°C after which the bacteria was pelleted and a crude lysate prepared. The chitin column was run with the crude lysate at room temperature as described in Materials and Methods. 40 µLs of the crude lysate were loaded onto a SDS-15% polyacrylamide gel, transferred to nitrocellulose membrane, and immunochemically stained with anti-HDAC2 polyclonal antibodies. Pink arrow: cellular endogenous HDAC2; Green arrow: pTYB1-TAT-HDAC2-C-terminal; Red arrow: TAT-HDAC2-C-terminal. *Molecular Mass Marker shown on right.*

Purification of pTYB1-TAT-HDAC2-C-terminal and Intein tag binding.

A chitin column was preformed a second time with a change in growth conditions to test for better growth conditions for the pTYB1-TAT-HDAC2-C-terminal construct. Changes included growing bacteria in TB media, filtering the crude lysate through at 0.2 µm filter, and running the column at 4°C. Fig 5 shows pTYB1-TAT-HDAC2-C-terminal

present in the crude cell lysate and in the flow-through of the column after the addition of the lysate. The protein was not detected on the chitin beads or in any eluted fractions. These results indicate that the construct is present, but not attaching to the chitin resin in the column. There are two possible explanations for this result, either the protein is not folding properly after induction with IPTG and thus, affecting the conformation of the intein tag and the chitin-binding domain or the chitin beads are defective.

Detection of chitin-binding domain

To determine whether the protein was denatured in the bacteria, a chitin column was attempted a third time and a western blot run on the samples. pTYB1-TAT-HDAC2-C-terminal was transformed in BL21 bacteria and growing in TB media. The culture was induced with 0.6 mM IPTG and then a crude lysate prepared as described in Materials and Methods. Fig 6 shows a western blot completed with the anti-chitin-binding domain antibody. This antibody tests specifically for the chitin-binding domain (CBD) in the Intein tag of the pTYB1 vector. The CBD was present in the crude lysate and the flow-through of the column after the addition of the lysate. A similar western blot was completed on the samples with a HDAC2 polyclonal antibody (results not shown). These results indicate that the protein is not denatured since both the HDAC2 and CBD elements of the construct were detected. Fig 6 displays that the protein construct did not attach to the chitin beads before the addition of DTT to induce the intein tag self-cleavage nor is it present in the eluted fractions. These data further the notion that the protein has not folded correctly or the chitin beads are defective.

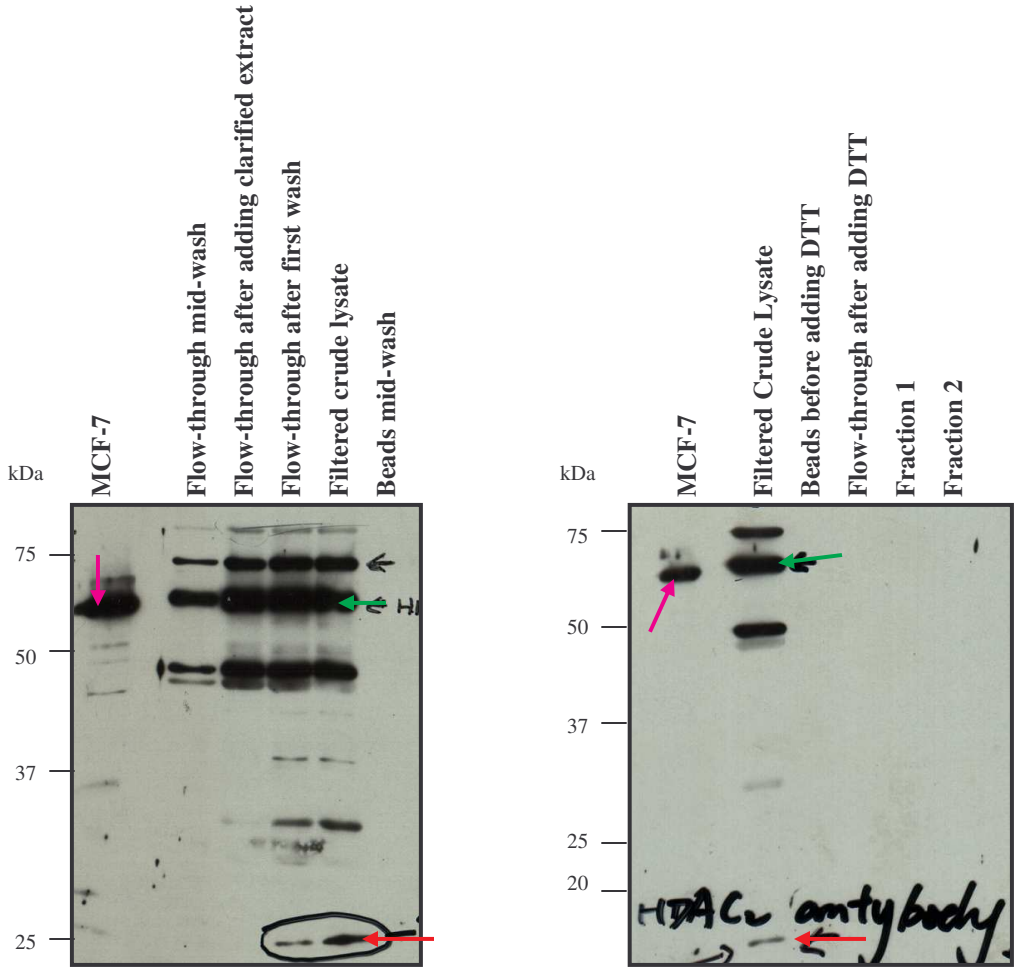


Fig 5: pTYB1-TAT-HDAC2-C-terminal present in crude lysate but does not bind to Chitin resin.

MCF-7 nuclei were used as controls for HDAC2 and Chitin. BL21 bacteria transformed with 534 was grown in 2 liters of TB media in at 37°C until the culture reached OD_{600} 0.493. The culture was induced with 0.5 mM IPTG for 4 hours at 37°C after which the bacteria was pelleted and a crude lysate prepared. The lysate was filtered through a 0.2 µm filter (get name and type) and ran through a chitin column at 4°C as described in Materials and Methods. 10 µLs of sample were taken from each step of the column and loaded onto a SDS-10% polyacrylamide gel, transferred to nitrocellulose membrane, and immunochemically stained with anti-HDAC2 polyclonal antibodies. Pink arrow: cellular endogenous HDAC2; Green arrow: pTYB1-TAT-HDAC2-C-terminal; Red arrow: TAT-HDAC2. *Molecular Mass Marker shown on right.*

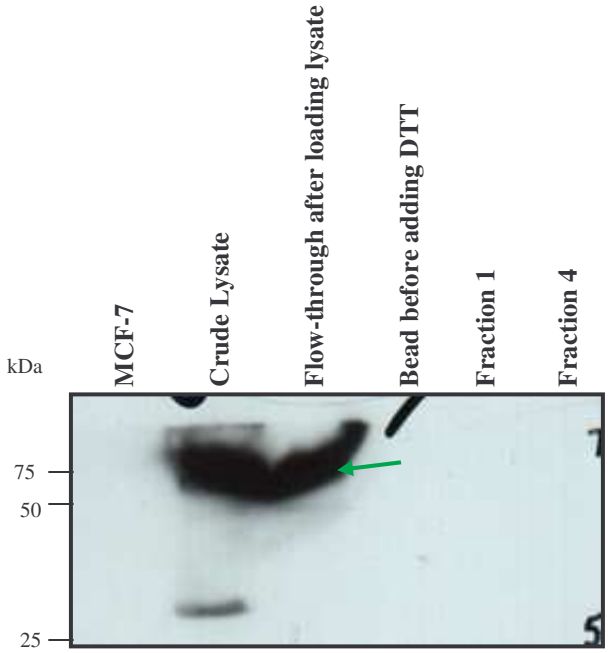


Fig 6: Determination of Chitin-binding domain.

MCF-7 nuclei were used as controls for HDAC2. BL21 bacteria transformed with 534 was grown in 1 liter of TB media at 37°C until the culture reached OD_{600} 0.507A. The culture was induced with 0.3 mM IPTG overnight at 37°C after which the bacteria were pelleted and a crude lysate prepared. The crude lystae was run through a chitin column as described in Materials and Methods. 10 µLs of each sample were loaded onto a SDS-15% polyacrylamide gel, transferred to nitrocellulose membrane, and immunochemically stained with anti-chitin-binding domain antibodies. Green arrow: pTYB1-TAT-HDAC2-C-terminal. *Molecular Mass Marker shown on right.*

Failure of pTYB1-Bax to attach to chitin column

The IMPACT-CN system had been previously used by another member of the Manitoba Institute of Cell Biology successfully. The pTYB1-Bax protein was donated by Evan Buoy of Dr. Spencer Gibson's lab to use as a control for pTYB1-TAT-HDAC2-C-terminal. New England Biolabs recommended attempting induction of the proteins with IPTG at a lower temperature over night and sent new chitin resin to use in the chitin column. pTYB1-TAT-HDAC2-C-terminal and pTYB1-Bax were each grown in a liter of TB media and then induced with 0.3 mM IPTG at 21°C overnight and then a crude lysate prepared. Separate chitin columns were run with each sample as described in Materials and Methods. Fig 7*(A)* detected HDAC2 in the HEK293 and BL21 crude cell lysate with

the pTYB1-TAT-HDAC2-C-terminal construct but not in pTYB1-Bax. The pTYB1-Bax has a protein size of 76 kDa. Since HDAC2 is not present in Bax or in *E. coli*, this result was expected, although, some non-specific bands were detected. Furthermore, a CBD (Fig 7*(B)*) was detected in both protein lysates and in the column flow through after the addition of the lysate. However, neither protein construct was detected in the eluted fractions of the columns, demonstrating that both pTYB1-Bax and pTYB1-TAT-HDAC2-C-terminal failed to attach to the chitin beads. A change in the growth conditions through lowering the temperature of induction and new chitin resin still did not yield successful results.

Affects of TSA on MCF-7 breast cancer cells.

Sekhavat (2007) reported that TSA acts as a competitive inhibitor of HDAC2. HDAC inhibition by TSA targets a range of HDACs at specific locations, however, it has been found to have an affect on p21. As a result, it suits this experiment as the control to the pTYB1-TAT-HDAC2-C-terminal construct. MCF-7 breast cancer cells ($1 \text{ X } 10^5$) were plated on Corning cell culture dishes until they reached 75-85% confluence within 6 days. Cells were incubated with 500 ng/mL TSA (Davie and Spencer 2001) and absolute ethanol and observed every 0.5, 6, 12, 24, 48, and 72h. Fig 8 *(A)* demonstrates the affects of TSA on breast cancer cells. After 12h, cells begin to reveal changes in morphology. Cell shape begins to change: cells shrink and round up or begin to elongate. Cell nuclei became less visible and cells began to die (growth curves were not completed). Lipid droplet accumulation can be vaguely viewed in the cells. Fig 8*(B)* demonstrates the control MCF-7 cells incubated with absolute ethanol. Some change in cell shape was observed, however, it was not as drastic or noticeable as in Fig 8*(A)*. Alive cells at 72h

continued to be round and easily discernable. The toxic effects seen in the cells incubated with TSA were not observed.

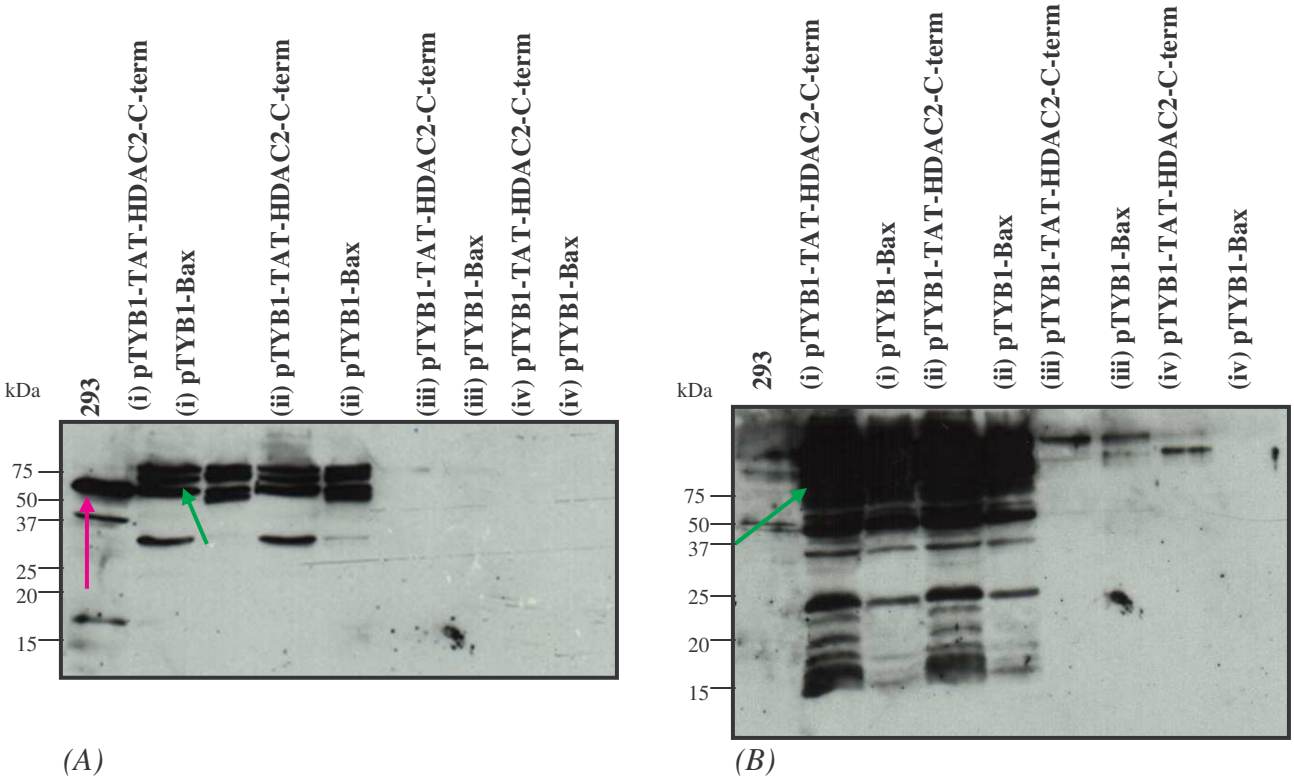


Fig 7: pTYB1-Bax protein control for pTYB1-TAT-HDAC2-C-terminal construct.

HEK293 nuclei were used as controls for HDAC2. BL21 bacteria transformed with pTYB1-TAT-HDAC2-C-terminal and pTYB1-Bax were grown in 1 liter of TB media in separate flasks at 37°C until the cultures reached an OD_{600} of 0.400-0.600. The cultures were induced with 0.3mM IPTG overnight at 21°C after which the bacteria were pelleted and a crude lysate prepared as described in Materials and Methods. 15 µLs of the crude lysate were loaded onto a SDS-15% polyacrylamide gel, transferred to nitrocellulose membrane, and immunochemically stained with (A) anti-HDAC2 polyclonal and (B) anti-chitin-binding domain antibodies. (i) Crude cell lysate (ii) Flow-through from addition of crude lysate to chitin column (iii) Elution 1 (iv) Elution 4. Pink arrow: cellular endogenous HDAC2; Green arrow: Fusion Protein. *Molecular Mass Marker shown on right.*

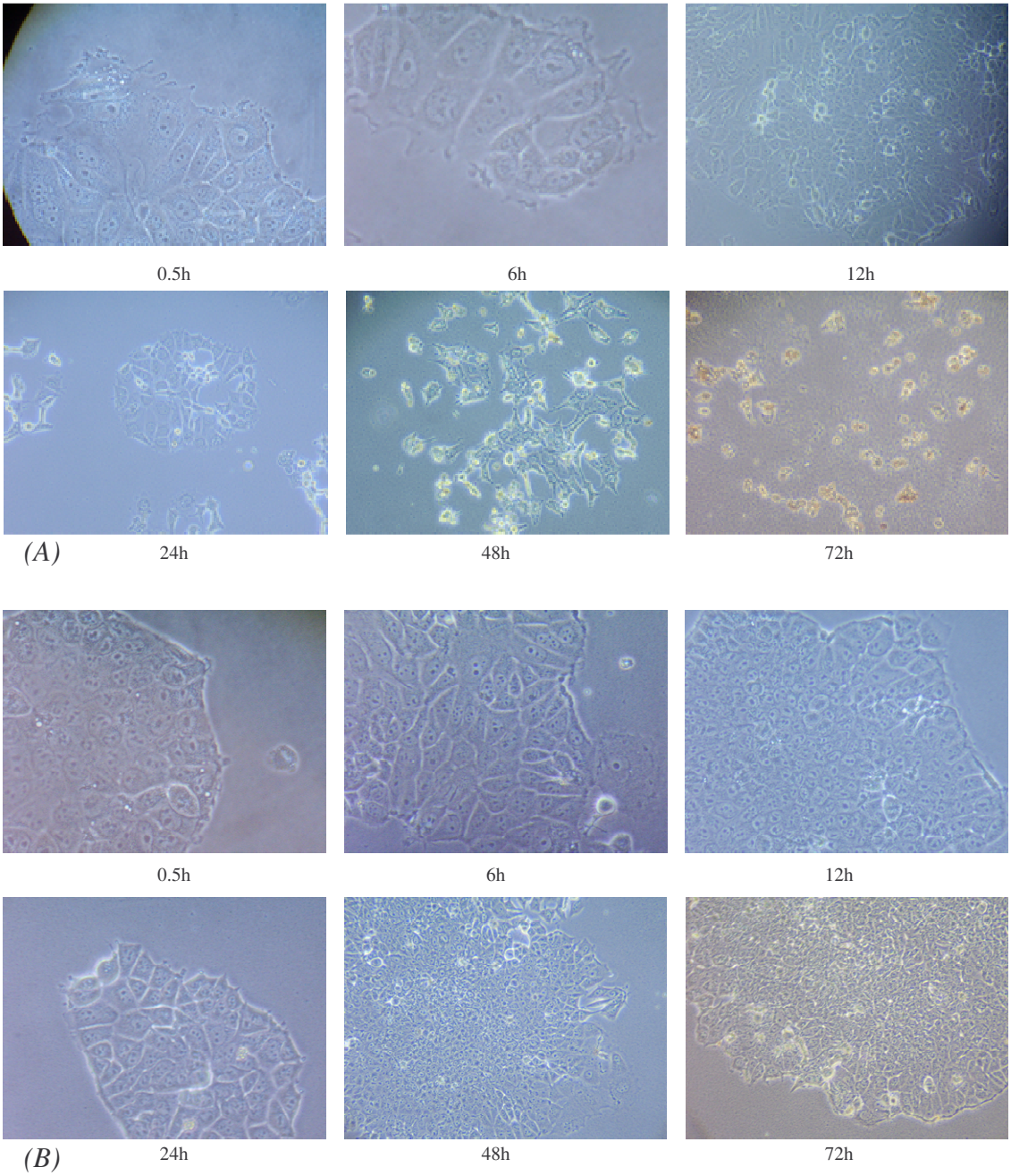


Fig 8: MCF-7 breast cancer cells treated with TSA and EtOH over 72h.

MCF-7 cells (1 X 10^5) were plated in DMEM, 5% FBS and incubated with (a) 500 ng TSA or (b) 100% absolute ethanol over 72h. Visible changes in morphology of cells were observed. *Note*: magnification for all pictures is not the same

Discussion

The hypothesis of this experiment could not to be tested because of the inability to successfully isolate and purify the pTYB1-TAT-HDAC2-C-terminal construct.

The Intein Mediated Purification with an Affinity Chitin-binding tag (IMPACT-CN) protein purification system utilizes the inducible self-cleavage activity of a protein splicing element, in this case, intein (New England Biolabs). The system allows the purification of a desired protein in a single chromatographic step without the use of a protease. The intein is from the *Saccharomyces cerevisiae* VMA1 and contains a chitin binding domain which has an high affinity to the chitin resin in a column. In the presence of thiols, such as DTT, the intein tag undergoes specific self-cleavage and releases the target protein from the chitin bound intein tag (See Appendix A) (New England Biolabs). The pTYB vectors used with the IMPACT-CN system are to allow for the cloning and expression of recombinant proteins in *E. coli*. pTYB1 is 7,477 bp in size and fuses to the intein tag to the C-terminus of the target protein. The pTYB vectors contain the T7/*lac* promoter which allows for stringent control of the fusion gene expression induced only by IPTG. Given these qualities and simple methodology, the IMPACT-CN system seemed to be an ideal candidate for a protein purification system for my experiment.

Figs 3-7 demonstrate an inability to successfully purify the TAT-HDAC2 fusion protein. Two main reasons emerge for the explanation of the poor binding of the intein tag to the chitin resin. Either the protein did not fold properly after induction with IPTG or that the CBD in the intein tag is deficient. IMPACT-CN lists seven possible reasons for poor protein yield:

- *E. coli* strain

- Culture conditions (temperature, aeration, cell density)
- Induction and protein expression conditions (temperature, time, and concentration of IPTG)
- Expression level of fusion protein
- Solubility of fusion protein
- Efficiently of thiol-induced cleavage
- Solubility of target protein after the cleavage

In order to address and minimize the above reasons for a poor protein yield different trouble-shooting steps were attempted. Becker-Hapak and Dowdy (2003) found that in some instances a construct expressed poorly in LB but more abundantly in other media such as TB. As a result, after the failure of the first chitin column to purify the protein successfully, the media to grow BL21 was changed from LB to TB in hopes that it would provide better growth conditions for the protein expression and induction.

Furthermore, the IMPACT-CN protein purification protocol recommends that the temperature of induction with IPTG be 30°C for 3 hours, 22-25°C for 6 hours or 12-15°C for overnight. The temperature of the shaker in the lab cannot be reduced to 15°C unless placed in the cold room, which was physically impossible. Protein induction to allow for proper protein folding was attempted at differing temperatures (Fig 4 & Fig 7) such as 37°C overnight or for 3-4 hours as well as at 21°C overnight (Fig 4, 6, & 7). Different induction temperatures and concentrations of IPTG did not result in successful protein purification.

In order to test the effectiveness of the chitin resin, new chitin beads were purchased from New England Biolabs. Furthermore, the chitin column was attempted at room temperature and at 4°C to test whether temperature would affect the chitin resin

affinity. As Fig 5, 6 and 7 demonstrate, a change in temperature did not affect the chitin binding on the affinity column.

To ensure that the failure of the protein purification system was not due to poorly or incorrectly made solutions, all buffers were made fresh twice. The columns were also prepared with varying levels of NaCl in the column buffer from 0-100 M. Salt concentration in the buffers did not appear to increase the level of affinity between pTYB1-TAT-HDAC2-C-terminal and the chitin resin.

Finally, a protein that had been purified using the IMPACT-CN system was given to the JRD lab from Evan Buoy in Spencer Gibson's lab at the Manitoba Institute of Cell Biology (FIG 7). pTYB1-Bax had been previously purified successfully using the IMPACT-CN protein purification system. The construct was used as a control for pTYB1-TAT-HDAC2-C-terminal. Both constructs were transformed and expressed in BL21 bacteria and crude lysates prepared as described in Materials and Methods. Fig 7 shows that pTYB1-Bax and pTYB1-TAT-HDAC2-C-terminal failed to attach to the chitin resin. In essence, these results indicate that more tests need to be completed in order to determine the reason for the failure of the purification system. The chitin resin affinity was not tested using an intein tag that demonstrated that the chitin beads were not deficient. Furthermore, more tests with changes in growth conditions need to be attempted. My tests did not conclusively prove that the protein is mis-folding or that the chitin resin is indeed deficient.

Following the three months of trouble-shooting, it was determined that another protein purification strategy may work better for this experiment. Some options include the HexaHistidine (HIS), glutathione S-transferase (GST) or Maltose-binding protein

(MBP) tags (Lichty and others 2005). All three tags bind to small molecules (amylase, immobilized glutathione or immobilized colbalt or nickel ions) and can be eluted from the resin by competition with an analog (imidazole for HIS, glutathione for GST and maltose for MBP) (Lichty and others 2005). These tags have been used in the lab consistently without failure and might prove to be more successful than the IMPACT-CN protein purification system.

Once the protein is successfully purified, large-scale purification of the TAT-fusion proteins will take place in order to acquire large amounts of the protein. Then the Tat-HDAC2 fusion proteins will be transduced into cells *in vitro* in the same fashion they were incubated with TSA in Fig 8. The effectiveness and ability of the TAT-HDAC2-C-terminal fusion protein to act as a competitive inhibitor to cellular HDAC2 and bind to RbAp48 thereby disrupting the Sin3A and NuRD corepressor complexes will be determined by visually examining the cells for morphological changes. Cells characteristics such as shape, size, growth or differentiation will be observed. Cells that continue to grow and live will indicate the ineffectiveness of the fusion protein. Cell death will indicate the activation of the p21 protein, and therefore the success of HDAC inhibition by the TAT-HDAC2 fusion protein. Cells will be determined dead or alive through visual observation and immunofluorescent staining methods. Furthermore, TSA-induced cell death triggers cytochrome c release from mitochondria and can be tested for by immunofluorescence assays using an anti-cytochrome c antibody (Henderson and others 2003). Chromatin immunoprecipitation (ChIP) assays could test the acetylation of histone proteins located at specific promoter and coding regions in the genome. Using an antibody for acetylated histones (e.g. antibody against acetylated K9, K14 H3), a ChIP

assay would identify acetylation differences between the coding and promoter regions in the cell, thereby providing evidence of the effectiveness of the TAT-HDAC2 protein to affect the targeted phosphorylated HDAC2 functions. To test for effective disruption of the Sin3A and NuRD corepressor complexes, co-immunoprecipitation (co-IP) assays will be used to determine the integrity of the protein complexes. Using specific antibodies to components of the NuRD (e.g. MBD3) and Sin3A (e.g. Sin3) corepressor complexes, immunoprecipitation experiments will isolate the NuRD and Sin3A in control cells and cells transduced with TAT-HDAC2. Western blot analyses of the immunoprecipitates would determine whether HDAC1, HDAC2 and RbAp48 were present or absent in the immunoprecipitated complex, providing insights into whether the complex was dissociated in the presence of the TAT-HDAC2 fusion protein.

In order to improve the protein expression, the possibility of changing the TAT-protein transduction domain to a different cell penetrating peptide also exists. As discussed in the introduction, the intracellular delivery of proteins is difficult to achieve due to the plasma membrane. The third alpha helix of the Antennapedia homeodomain protein is a cell penetrating peptide and is termed Antp (Meade and Dowdy 2007). Antp contains a high density of basic charges similar to TAT (Meade and Dowdy 2007). Transportan and polyarginine are also commonly known and used CPPs. A study completed by Jones and others (2005) found that the uptake of the CPPs by three different cell lines (HeLa, A549 and CHO) ranged in magnitude with transportan and pholyarginine taken up most easily followed by antennapedia and then TAT. Furthermore, the study found that antennapedia is the least toxic CPP followed by TAT, transportan and finally polyarginine (Jones and others 2005). The study determined that antennapedia

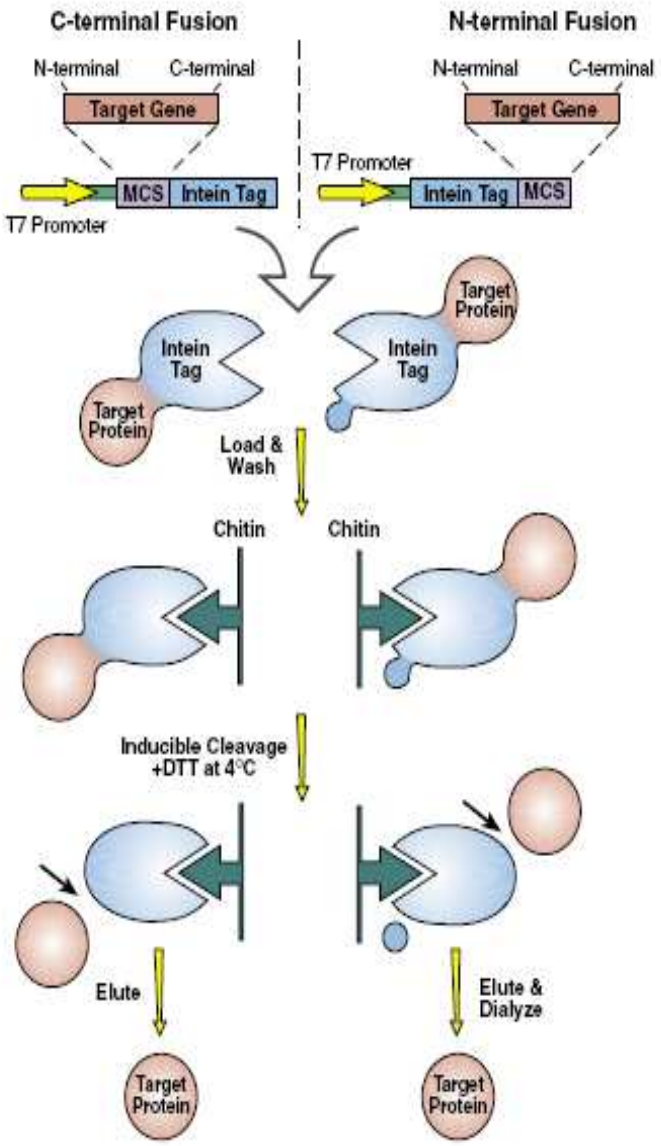
serves as the optimal CPP in comparison to the other three (Jones and others 2005). If toxicity and cellular uptake is found to be important in isolating and testing the TAT-HDAC2 protein, then using Antennapedia as the CPP rather than TAT may be a consideration.

Conclusions

- There is an emerging interest in HDAC inhibitors as cancer therapeutic agents
- Cell-penetrating peptides such as TAT have been employed in *in vivo* and *in vitro* delivery of biologically active peptide
- The hypothesis was not tested due to the unsuccessful purification of the TAT-HDAC2-C-terminal fusion protein using the IMPACT-CN protein purification system
- Possible reasons and alternative mechanisms were provided to purify the TAT-HDAC2-C-terminal fusion protein and test its ability to sequester RbAp48 and disrupt the Sin3A and NuRD corepressor complexes

Appendices

Appendix A. Schematic illustration of IMPACT-CN protein purification system



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