

The Possible Role for Histone Deacetylase Inhibition as a Radiosensitizer in Chordoma

Jake Griner

Major: Biochemistry and Molecular Biology

Advisor: Dr. Brittany Gasper

Abstract:

Chordoma is a rare primary spinal cancer which affects one in one million people and has an average survival time of seven years. Despite advances in surgical techniques and radiation therapy, approximately 30% of chordoma patients develop metastatic disease at some point. Local recurrence is a significant issue for case where the tumor cannot be completely removed, and may appear as soon as a few months post-surgery. For this reason, there is great clinical interest in finding effective therapies that can prevent or treat recurrent or metastatic tumors. In this project, it was determined that one out of three tested chordoma cell lines experienced significant radio-sensitization following exposure to vorinostat, an FDA approved pan-HDAC inhibitor. In the three cell lines tested, it does not appear that HDAC6 plays a major role in radiation response. While further testing is necessary, this data suggests that vorinostat in combination with radiation is a possible therapeutic option for chordoma and should be investigated.

Introduction:

Background on Chordoma

Chordoma is a relatively rare cancer of the skull and spine which accounts for approximately 3% of all bone malignancies,¹ but is one of the most common spinal tumors.² The incidence of chordoma is approximately 1 in 1,000,000 and average survival time is approximately seven years.³ There are currently no FDA approved systemic therapies for chordoma. Treatment of chordoma frequently begins with surgical resection of the primary

¹ Healey JH, Lane JM. Chordoma: a critical review of diagnosis and treatment. *Orthop Clin North Am* 1989; **20**: 417–26.

² Kelley, S. P., Ashford, R. U., Rao, A. S., & Dickson, R. A. (2007). Primary bone tumours of the spine: a 42-year survey from the Leeds Regional Bone Tumour Registry. *European Spine Journal*, *16*(3), 405–409. <http://doi.org/10.1007/s00586-006-0188-7>

³ M.L. McMaster, A.M. Goldstein, C.M. Bromley, N. Ishibe, and D.M. Parry, "Chordoma: incidence and survival patterns in the United States, 1973-1995.", *Cancer causes & control: CCC*, 2001.

tumor. Because primary tumors are usually slow growing, they are often discovered after they have grown quite large, and because of their locally invasive tendencies, the preferred surgical technique for chordoma is an *en bloc* resection — the removal of the tumor with no dissection. This was established to be the best surgical protocol in the late 1970's and continues to be widely used.⁴ The implications of this are profound; time before recurrence for patients who have a successful *en bloc* resection can be more than ten years, but only eight months on average for patients who do not.⁵ Unfortunately, because of tumor location, size, and the level of invasiveness at the time of discovery, *en bloc* resection is only possible in less than half of all chordoma patients.⁶

Even with a successful *en bloc* resection, there is still a possibility for local recurrence. This is attributed to micro-skip metastasis, a recently discovered phenomenon in which the tissue immediately surrounding the tumor appears to be cancer free, but looking farther beyond the tumor margin reveals microscopic nodules of metastatic chordoma.⁷ To reduce the risk of local recurrence, chordoma surgeries are often followed by adjuvant radiation therapy to the tumor bed.⁸ A 2014 study by DeLaney *et al.* showed that with proton beam therapy dose of ~72 Gray, 85% of patients have local control at five years.⁹ Despite the significant progress made in surgical technique and radiation therapy, recurrence is still common. Less than 50% of chordoma

⁴ Stener B, Gunterberg B. High amputation of the sacrum for extirpation of tumors. Principles and technique. *Spine* 1978; **3**: 351–66.

⁵ Fuchs B, Dickey ID, Yaszemski MJ, Inwards CY, Sim FH. Operative management of sacral chordoma. *J Bone Joint Surg Am* 2005; **87**: 2211–16.

⁶ Bjornsson J, Wold LE, Ebersold MJ, Laws ER. Chordoma of the mobile spine. A clinicopathologic analysis of 40 patients. *Cancer* 1993; **71**: 735–40.

⁷ Akiyama, T., Ogura, K., Gokita, T., Tsukushi, S., Iwata, S., Nakamura, T., . . . Yamaguchi, T. (2017). Analysis of the Infiltrative Features of Chordoma: The Relationship Between Micro-Skip Metastasis and Postoperative Outcomes. *Annals of Surgical Oncology*, 25(4), 912-919. doi:10.1245/s10434-017-6268-6

⁸ Walcott, B. P., Nahed, B. V., Mohyeldin, A., Coumans, J. V., Kahle, K. T., & Ferreira, M. J. (2012). Chordoma: current concepts, management, and future directions. *The lancet oncology*, 13(2), e69-e76.

⁹ DeLaney, T. F., Liebsch, N. J., Pedlow, F. X., Adams, J., Weyman, E. A., Yeap, B. Y., ... & Chen, Y. L. (2014). Long-term results of Phase II study of high dose photon/proton radiotherapy in the management of spine chordomas, chondrosarcomas, and other sarcomas. *Journal of surgical oncology*, 110(2), 115-122.

patients have complete tumor resection, making local recurrence an immediate problem. Proton therapy has delayed the onset of recurrence, but it is nevertheless a common issue for patients in the years following treatment. Additionally, radiation therapy is only able to treat local disease — not metastatic disease. Metastatic disease is still a major issue for chordoma patients, as approximately 30% of all chordomas metastasize.¹⁰ For this reason, effective systemic therapies are essential to treat advanced chordoma. Comprehensive studies have unfortunately shown that traditional cytotoxic chemotherapies are largely ineffective due to the slow growing nature typical of chordoma. Because of the lack of available systemic therapies, average survival time for patients with metastatic disease is less than three years.¹¹

Effective treatments for the management of advanced chordoma are clearly needed. One significant approach to the treatment of recurrent or metastatic chordoma is through vaccination to the brachyury antigen. Brachyury is a nuclear transcription factor expressed in >98% of chordomas, but is not expressed in normal tissue.¹² In a Phase I clinical trial, the brachyury vaccine was moderately effective with 2/5 patients having tumor shrinkage or stabilization.¹³ The brachyury vaccine is now in a phase II clinical trial, which is comparing radiation alone versus vaccine + radiation for locally advanced chordoma. A variety of other therapeutic strategies for the management of recurrent and metastatic chordoma are currently underway. These include a CDK4/6 inhibitor, palbociclib, which is undergoing a phase II trial in Heidelberg, Germany, and radiation combined with a PD-1 inhibitor at Memorial Sloan Kettering and Johns Hopkins. Both

¹⁰ Gay, E., & Sekhar, L. (1995). Chordomas and chondrosarcomas of the cranial base: results and follow-up of 60 patients. *Neurosurgery*, 5(36), 887-896. Retrieved March 24, 2017.

¹¹ Bjornsson J, Wold LE, Ebersold MJ, Laws ER. Chordoma of the mobile spine. A clinicopathologic analysis of 40 patients. *Cancer* 1993; **71**: 735-40.

¹² Hamilton D, H, David J, M, Dominguez C, Palena C, Development of Cancer Vaccines Targeting Brachyury, a Transcription Factor Associated with Tumor Epithelial-Mesenchymal Transition. *Cells Tissues Organs* 2017;203:128-138

¹³ Heery, C. R., Singh, H., Marte, J. L., Madan, R. A., O'Sullivan Coyne, G. H., Farsaci, B., ... & Gulley, J. L. (2014). NCI experience using yeast-brachyury vaccine (GI-6301) in patients (pts) with advanced chordoma.

of these trials, and nearly all of the planned studies for advanced chordoma, are predicated on the disease recurrence. They are addressing treatment options when the disease returns. The aim of this study, however, is slightly different. The goal of this project is to determine if a radiosensitizing agent can prevent or significantly delay disease recurrence.

Histones and Histone Deacetylases

Histones are a class of small nuclear proteins which form octamers around which DNA wraps to form nucleosomes, the base level of chromatin organization. Histones can be acetylated on lysine residues, a modification which functions to activate the genes wrapped around that histone. Gene activation and inactivation is critical to cellular function and response to stress, and histone acetylation is critical to gene regulation. This is accomplished through two complementary groups of enzymes, histone acetyltransferases and histone deacetylases (HDAC). Histone deacetylases function by removing the acetyl group from a lysine residue on the histone, thus inactivating the gene. The family of HDACs contains 11 members (HDAC1-11) which can be grouped into four major classes: I, IIA, IIB, and IV depending on their amino acid sequence. It is worth noting that the name ‘histone deacetylase’ is a bit of a misnomer, as these proteins have targets which are not histones. This is the case in HDAC6, which we will return to later.

In many cancer types, overexpression of one or more HDACs contributes to disease progression.¹⁴ Histone deacetylases appear to contribute to cancer progression by a few mechanisms. Briefly, this can include increasing the ‘barrier of entry’ into programmed cell death,¹⁵ increasing expression of p-glycoprotein efflux pump which detoxifies cells of

¹⁴ Nakagawa,M., Oda,Y., Eguchi,T., Aishima,S., Yao,T., Hosoi,F., Basaki,Y., Ono,M., Kuwano,M., Tanaka,M. *et al.* (2007) Expression profile of class I histone deacetylases in human cancer tissues. *Oncol. Rep.*, **18**, 769–774.

¹⁵ De Schepper,S., Bruwiere,H., Verhulst,T., Steller,U., Andries,L., Wouters,W., Janicot,M., Arts,J. and Van Heusden,J. (2003) Inhibition of histone deacetylases by chlamydocin induces apoptosis and proteasome-mediated degradation of survivin. *J. Pharmacol. Exp. Ther.*, **304**, 881–888.

chemotherapy,¹⁶ and compacting chromatin thus reducing the efficacy of genotoxins like etoposide.¹⁷ Most notable for this project is the role of HDACs in DNA damage repair. Ionizing radiation derives its therapeutic usefulness through inducing damage to DNA, most prominently double stranded breaks. If left unrepaired, DNA damage prevents genome replication and even normal function, resulting in death. Cells have, therefore, evolved remarkable machinery which repair DNA damage. The method of repair is dependent on the nature of the lesion, which can be loosely grouped into the following categories: base excision repair (BER), nucleotide excision repair, mismatch repair, homologous recombination (HR), and non-homologous end joining (NHEJ). The most common lesions resulting from radiation therapy are double stranded breaks.¹⁸ Double stranded breaks are often repaired via NHEJ or HR, depending on the nature of the break.

Non-Homologous End Joining

Doubled-strand DNA breaks can be repaired via two pathways: HR or NHEJ. Because of the complexity of lesions induced by ionizing radiation, repair via the NHEJ pathway is the most common.¹⁹ The complex mechanisms behind NHEJ are outside of the scope of this project, however, the role of HDACs in the signaling cascade that initiates NHEJ is of great interest. It is known that a crucial early step is the detection of DSBs by the MRN complex which consists of Mre11, Rad50, and Nbs1.²⁰ In the case of NHEJ, the MRN complex then recruits the kinase

¹⁶ Wang H, Huang C, Zhao L, et al. Histone deacetylase inhibitors regulate P-gp expression in colorectal cancer via transcriptional activation and mRNA stabilization. *Oncotarget*. 2016;7(31):49848-49858. doi:10.18632/oncotarget.10488.

¹⁷ Kim,M.S., Blake,M., Baek,J.H., Kohlhagen,G., Pommier,Y. and Carrier,F. (2003) Inhibition of histone deacetylase increases cytotoxicity to anticancer drugs targeting DNA. *Cancer Res.*, **63**, 7291–7300.

¹⁸ Waters,C.A., Strande,N.T., Wyatt,D.W., Pryor,J.M. and Ramsden,D.A. (2014) Nonhomologous end joining: a good solution for bad ends. *DNA Repair (Amst)*, **17**, 39–51.

¹⁹ Ibid.

²⁰ Symington,L.S. and Gautier,J. (2011) Double-strand break end resection and repair pathway choice. *Annu. Rev. Genet.*, **45**, 247–271.

ATM. ATM has a number of roles in DNA damage repair, but, for the sake of simplicity, we shall focus on the phosphorylation of histone H2AX on serine-139.²¹ Phosphorylated H2AX, abbreviated γ H2AX, is a well-studied marker of DSBs. To illustrate the speed at which these events progress, γ H2Ax can be detected less than 20 seconds after irradiation.²² The binding of the Ku70/80 heterodimer is another critical step in NHEJ. Firstly, their binding protects the DNA from further degradation by endogenous exonucleases.²³ Ku70/80 also provides a scaffold for the next step of NHEJ, the binding of DNA-PKcs,²⁴ which orchestrate the remaining steps of NHEJ.

The Role of HDAC in NHEJ

Now that the process of NHEJ has been briefly described, let us turn to the role that histone deacetylases play in this process. NHEJ requires remodeling of chromatin to allow repair proteins to access the damaged DNA. HDAC1 and 2 have been shown to deacetylate H3K56Ac and H4K16Ac and promote NHEJ.²⁵ The binding of the Ku70/80 heterodimer is also regulated by HDACs. Acetylated Ku70 has a lower affinity for DNA, therefore its deacetylation is critical to formation of the Ku70/80 heterodimer.²⁶ It has been shown that inhibition of HDAC1,2, and 3 causes increased sensitivity to chemotherapeutics because of decreased Ku70/80 formation and decreased DNA repair.²⁷

²¹ Burma,S., Chen,B.P., Murphy,M., Kurimasa,A. and Chen,D.J. (2001) ATM phosphorylates histone H2AX in response to DNA double-strand breaks. *J. Biol. Chem.*, **276**, 42462–42467.

²² Rogakou, E. P., Pilch, D. R., Orr, A. H., Ivanova, V. S., & Bonner, W. M. (1998). DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *Journal of biological chemistry*, 273(10), 5858-5868.

²³ Downs,J.A. and Jackson,S.P. (2004) A means to a DNA end: The many roles of Ku. *Nat. Rev. Mol. Cell Biol.*, **5**, 367–378.

²⁴ Ibid.

²⁵ Miller,K.M., Tjeertes,J.V., Coates,J., Legube,G., Polo,S.E., Britton,S. and Jackson,S.P. (2010) Human HDAC1 and HDAC2 function in the DNA-damage response to promote DNA nonhomologous end-joining. *Nat. Struct. Mol. Biol.*, **17**, 1144–1151.

²⁶ Chen,C.S., Wang,Y.C., Yang,H.C., Huang,P.H., Kulp,S.K., Yang,C.C., Lu,Y.S., Matsuyama,S., Chen,C.Y. and Chen,C.S. (2007) Histone deacetylase inhibitors sensitize prostate cancer cells to agents that produce DNA double-strand breaks by targeting Ku70 acetylation. *Cancer Res.*, **67**, 5318–5327.

²⁷ Zhang,F., Zhang,T., Teng,Z.H., Zhang,R., Wang,J.B. and Mei,Q.B. (2009) Sensitization to gamma-irradiation-induced cell cycle arrest and apoptosis by the histone deacetylase inhibitor trichostatin A in non-small cell lung cancer (NSCLC) cells. *Cancer Biol. Ther.*, **8**, 823–831. **And** Blattmann,C., Oertel,S., Ehemann,V., Thiemann,M.,

The Role of HDAC6

A 2013 paper by Scheipl *et al.* looked at the expression of the various HDACs in 50 chordoma biopsies and found that HDAC6 was the most prevalently expressed.²⁸ They also found that HDAC inhibition by vorinostat decreased cell viability and increased apoptosis in a chordoma cell line.²⁹ Unlike nearly all of the other HDACs, which are almost exclusively localized to the nucleus, HDAC6 is primarily a cytoplasmic protein. HDAC6 functions primary to deacetylate two non-histone target proteins: α -tubulin and Hsp90. α -tubulin is a cytoskeletal protein, and the discovery of this interaction has contributed to our understanding of the role that HDAC6 plays in cancer metastasis. More interestingly, for the purpose of this project, is the interaction between HDAC6 and Hsp90.

It has been established that Hsp90 is a substrate of HDAC6, and that HDAC6 inhibition leads to hyperacetylation of Hsp90.³⁰ Hsp90's role as a chaperone protein is tightly regulated by a number of posttranslational modifications. The role of acetylation and deacetylation in the function of Hsp90 remain a subject of study, however it appears that hyperacetylated Hsp90 is less effective at repairing DNA. The mechanism by which Hsp90 acetylation or inhibition induces radiation sensitivity is still unknown. However, there is abundant functional data showing that Hsp90 plays a critical role in radiation response. A 2010 paper by Namdar *et al.* showed that HDAC6 inhibition greatly reduced the ability of prostate cancer cells to repair DNA

Huber, P. E., Bischof, M., Witt, O., Deubzer, H. E., Kulozik, A. E., Debus, J. *et al.* (2010) Enhancement of radiation response in osteosarcoma and rhabdomyosarcoma cell lines by histone deacetylase inhibition. *Int. J. Radiat. Oncol. Biol. Phys.*, 78, 237–245.

²⁸ Scheipl, S., Lohberger, B., Rinner, B., Froehlich, E. V., Beham, A., Quehenberger, F., ... & Liegl, B. (2013). Histone deacetylase inhibitors as potential therapeutic approaches for chordoma: an immunohistochemical and functional analysis. *Journal of Orthopaedic Research*, 31(12), 1999-2005.

²⁹ Ibid.

³⁰ Kovacs, J. J., Murphy, P. J., Gaillard, S., Zhao, X., Wu, J. T., Nicchitta, C. V., ... & Yao, T. P. (2005). HDAC6 regulates Hsp90 acetylation and chaperone-dependent activation of glucocorticoid receptor. *Molecular cell*, 18(5), 601-607.

damage induced by alkylating chemotherapy agents and decreased cell proliferation.³¹ Similarly, a 2006 paper Dote *et al.*³² and a 2016 paper by Kinzel *et al.*³³ showed radiosensitizing effects from Hsp90 inhibition.

Clinical Uses of HDAC Inhibition as a Radiosensitizer

In other cancer types, there have been a few small Phase I studies evaluating vorinostat as a radiosensitizer. These include a 2015 study by DuBois *et al.* which combined vorinostat with a radiopharmaceutical (¹³¹I-MIBG) for the treatment of relapsed or refractory neuroblastoma.³⁴ A 2014 Phase I trial by Shi *et al.* examined the efficacy of vorinostat when combined with whole-brain radiation for the treatment of metastatic Non-small Cell Lung Cancer (NSCLC).³⁵ Being phase I trials, the primary outcome for both studies was to determine the safety rather than efficacy. Both papers found the combination to be tolerable at around 300mg/day of vorinostat, which is slightly below the FDA approved 400 mg/day for cutaneous T-cell lymphoma.

Radiosensitization in Chordoma

In a small case study of five patients, razoxane, a topoisomerase II inhibitor, was given along with an average of 63 Gy of photon-based therapy to patients with chordoma. While no direct controls were used, and the sample size prevents a statistically meaningful conclusion, 3/5

³¹ Namdar, M., Perez, G., Ngo, L., & Marks, P. A. (2010). Selective inhibition of histone deacetylase 6 (HDAC6) induces DNA damage and sensitizes transformed cells to anticancer agents. *Proceedings of the National Academy of Sciences*, 107(46), 20003-20008.

³² Dote, Hideaki, et al. "Inhibition of hsp90 compromises the DNA damage response to radiation." *Cancer research* 66.18 (2006): 9211-9220.

³³ Kinzel, Linda et al. "A novel HSP90 inhibitor with reduced hepatotoxicity synergizes with radiotherapy to induce apoptosis, abrogate clonogenic survival, and improve tumor control in models of colorectal cancer" *Oncotarget* vol. 7,28 (2016): 43199-43219.

³⁴ DuBois, Steven G., et al. "Phase 1 Study of Vorinostat as a Radiation Sensitizer with ¹³¹I-Metaiodobenzylguanidine (¹³¹I-MIBG) for Patients with Relapsed or Refractory Neuroblastoma." *Clinical Cancer Research* (2015): clincanres-3240.

³⁵ Shi, Wenyin, et al. "Vorinostat as a radiosensitizer for brain metastasis: a phase I clinical trial." *Journal of neuro-oncology* 118.2 (2014): 313-319.

patients were alive at 10 years following treatment.³⁶ This appears to be a moderate improvement over the normal 10-year survival rate of 40%. In a more recent *in vitro* study, LB100, a protein phosphatase 2a inhibitor which prevents progression through the cell cycle, was combined with radiation. This data showed that LB100 + radiation increased γ H2Ax, a marker of DNA damage, and decreased cell proliferation.³⁷ While these two studies provide rationale for this project, they both have significant limitations. Razoxane significantly increases the chance of developing a secondary malignancy like leukemia because of its broad toxicities. LB100 is only in early clinical investigation, meaning it is years away from possible FDA approval and the side effects are yet unknown. This project takes a different approach to radiosensitization in chordoma: investigating a possible role for histone deacetylase (HDAC) inhibition as a radiosensitizer in chordoma. Vorinostat is a pan-HDAC inhibitor, meaning it inhibits all members of the HDAC Class I and II family, and is FDA approved for the treatment of cutaneous T cell lymphoma.

³⁶ Rhomberg, Walter, et al. "Combined radiotherapy and razoxane in the treatment of chondrosarcomas and chordomas." *Anticancer research* 26.3B (2006): 2407-2411.

³⁷ Hao, Shuyu, et al. "Protein phosphatase 2A inhibition enhances radiation sensitivity and reduces tumor growth in chordoma." *Neuro-oncology* 20.6 (2017): 799-809.

Methods:

Cell Lines:

This project utilized three distinct chordoma cell lines: CH22, UCH-2, and UCH-17M. These cell lines were chosen to represent the morphologic diversity seen in chordoma, and have different rates of cell division and different expression levels of various proteins such as brachyury and CD24. CH22 and UCH-2 are derived from locally recurrent sacral chordomas, while UCH-17M is derived from a soft tissue metastasis. The cell lines were kindly provided by the Chordoma Foundation (Durham, NC), and maintained according to ATCC protocols.

Drugs:

Vorinostat and tubacin were acquired from Med Chem Express (Monmouth Junction, NJ). Samples were dissolved in DMSO to a concentration of 10mM and stored at -20°C.

Cell Viability Assay:

To determine the effect of HDAC inhibition as a monotherapy, cells were cultured in a 96 well plate (2000 cells/well) and allowed to proliferate overnight. The following day, vorinostat, tubacin, or control (cell culture media) was added to final concentration of 10 uM, 5 uM, 1uM, or 0 uM. The cells were incubated for 24, 48, or 72 hours post drug addition, and then harvested for viability. Viability was assessed using the Promega CellTiter 96 Non-Radioactive Cell Proliferation Kit (Promega Corporation, Madison, WI). Each treatment condition was performed in a replicate of 4. Data was analyzed using Microsoft Excel and GraphPad Prism. Statistics were performed using the Kruskal-Wallis test and post hoc analysis between groups.

To determine the effect of HDAC inhibition in combination with radiotherapy. Each cell line was initially split into three groups: control/ no treatment, 2.5 uM vorinostat, and 2.5 uM tubacin. The cell lines were pretreated for 24 hours, before being harvested for irradiation. Each of the groups was then split in 2, which received either 10 Gy or 0 Gy (control). Cells were irradiated at Moffitt Cancer Center in Tampa, FL, and then returned to Florida Southern College. Each of the now 18 groups of cells were then counted using Trypan blue, their concentration adjusted, and seeded into 96 wellplates. Each group was in a replicate of 6. At 36 and 72 hours post irradiation, viability was analyzed as described above.

Western Blots

HDAC1, HDAC 6, Ku80, and γ H2Ax expression were analyzed from the same cell groups as described above. Briefly, cells were lysed using 50 mM Tris-HCL pH 7.4, 150 mM NaCl, 50 mM NaF, 1 mM EDTA, 10% NP-40, 1% Triton-X, and protease inhibitors. 100 uL of lysis buffer was used per 1 million cells, and protein was then quantified using a ThermoFisher NanoDrop. 20 ug of protein per lane was loaded into 12% SDS-PAGE gels, and protein was transferred to a nitrocellulose membrane. Membranes were incubated with the primary antibodies (Cell Signaling Technology, Danvers, MA) in TBS-T with 1% Milk overnight at 4 °C. After washing, the membranes were incubated with secondary antibodies for 1 hour at room temperature. The blots were developed using 1-Step NBT/BCIP substrate solution (ThermoFisher Scientific).

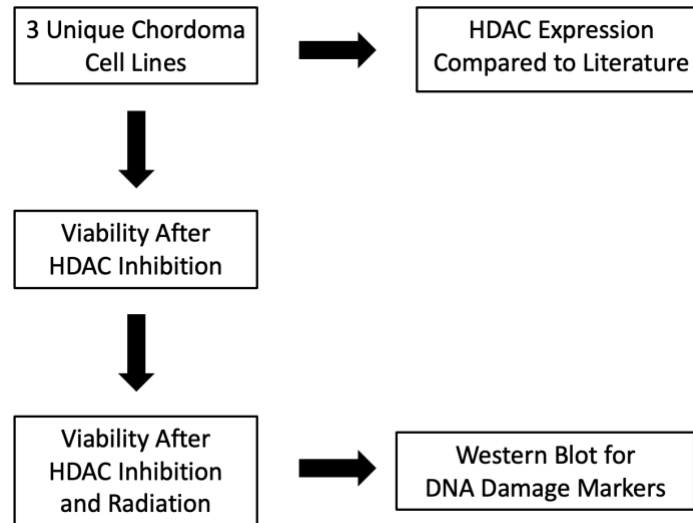


Figure 1. The workflow for the experiments described here.

Results

The original goal of this project was to determine the role of HDACs in DNA damage repair, and the possible therapeutic role of HDAC inhibition as a radiosensitizer in chordoma. There was literature evidence to suggest that HDAC inhibition does sensitize cancer cells to radiation, and two broadly defined mechanisms are presented. One is through the epigenetic changes induced by HDAC 1 and HDAC2 inhibition. Acetylated histones promote transcription of the genes wrapped around them, thus increasing their expression. It has been seen that HDAC inhibition restored the p53 tumor suppressor pathway in neuroblastoma.³⁸ In the context of radiation induced DNA damage, proper p53 expression is important for preventing progression through the cell cycle if unrepaired damage is present and even initiating apoptosis if the damage is irreparable. The relationship between HDAC inhibition and DNA repair protein expression, as

³⁸ Condorelli F, Gnemmi I, Vallario A, Genazzani AA, Canonico PL. Inhibitors of histone deacetylase (HDAC) restore the p53 pathway in neuroblastoma cells. *Br J Pharmacol.* 2007;153(4):657–668. doi:10.1038/sj.bjp.0707608

discussed in the introduction, is extremely complex and poorly understood. On one hand, HDAC inhibition could promote the expression of DNA repair genes and reduce cellular sensitivity to radiation. On the other hand, countless papers have observed that HDAC inhibition leads to transcriptional repression of repair-associated genes.³⁹

The second mechanism, investigated in this project, is the HDAC6 mediated role of Hsp90 in DNA damage repair. Tubacin specifically inhibits HDAC6, which appears to be required for the deacetylation and subsequent activation of Hsp90. Numerous papers have shown that Hsp90 inhibition has a radio-sensitizing and chemo-sensitizing effect in cancer cells, implicating it in DNA damage repair.⁴⁰ Furthermore, the 2013 paper by Schiepl *et al.* showed high levels of HDAC6 expression in chordoma, providing a possible explanation for its radioresistance.

³⁹ Roos WP, Krumm A. The multifaceted influence of histone deacetylases on DNA damage signalling and DNA repair. *Nucleic Acids Res.* 2016;44(21):10017–10030. doi:10.1093/nar/gkw922

⁴⁰ Pennisi R, Ascenzi P, di Masi A. Hsp90: A New Player in DNA Repair? [published correction appears in *Biomolecules.* 2016 Oct 18;6(4):]. *Biomolecules.* 2015;5(4):2589–2618. Published 2015 Oct 16. doi:10.3390/biom5042589

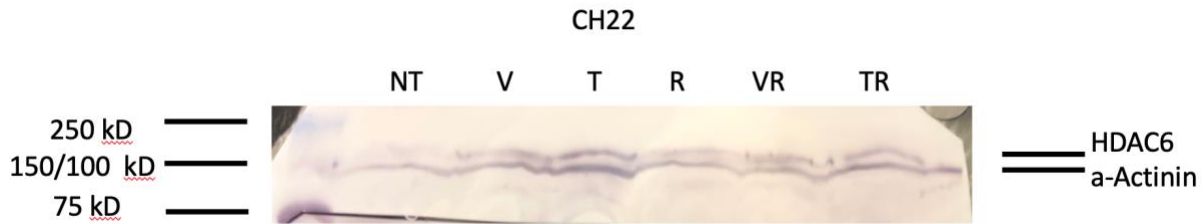


Figure 2. Expression of HDAC6 in the CH22 cell line. HDAC6 is expressed across the various treatment groups. It appears to be more highly expressed in treatment groups as compared to NT, but this claim cannot be backed up without band intensity quantification which could not be performed. NT= No Treatment, V= Vorinostat, T= Tubacin, R= Radiation (10 Gy), VR= Vorinostat (2.5 uM) + Radiation (10 Gy), TR= Tubacin (2.5 uM) + Radiation (10 Gy).

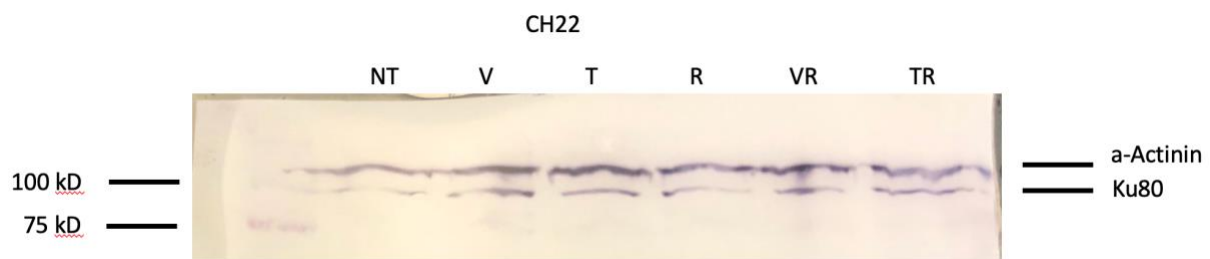


Figure 3. Expression of Ku80 in the CH22 cell line. Ku80, a protein involved in non-hologous end joining repair of DNA lesions, appears more highly expressed in the treatment groups as opposed to the NT group, but this claim cannot be backed up without band intensity quantification which could not be performed. NT= No Treatment, V= Vorinostat, T= Tubacin, R= Radiation (10 Gy), VR= Vorinostat (2.5 uM) + Radiation (10 Gy), TR= Tubacin (2.5 uM) + Radiation (10 Gy).

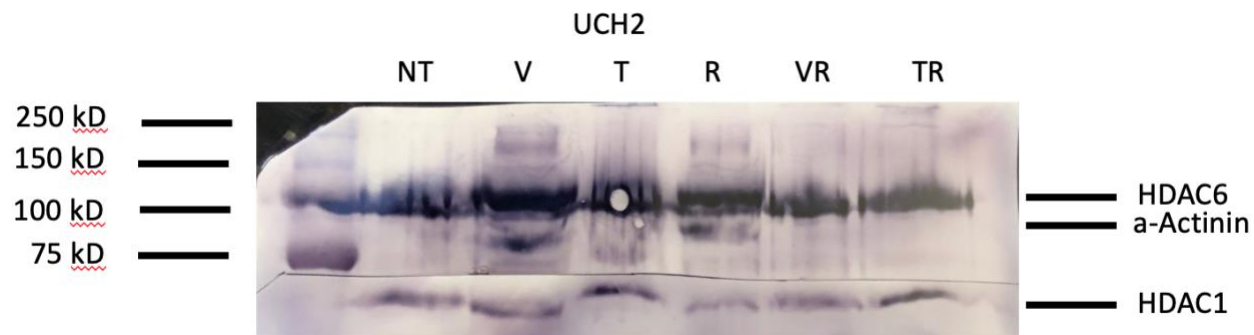


Figure 4. Expression of HDAC1 and HDAC6 in the UCH2 cell line. HDAC 1 and 6 appear to be expressed in the UCH2 cell line, although the broadening of the bands makes it difficult to distinguish HDAC6 from a-Actinin. NT= No Treatment, V= Vorinostat, T= Tubacin, R= Radiation (10 Gy), VR= Vorinostat (2.5 uM) + Radiation (10 Gy), TR= Tubacin (2.5 uM) + Radiation (10 Gy).

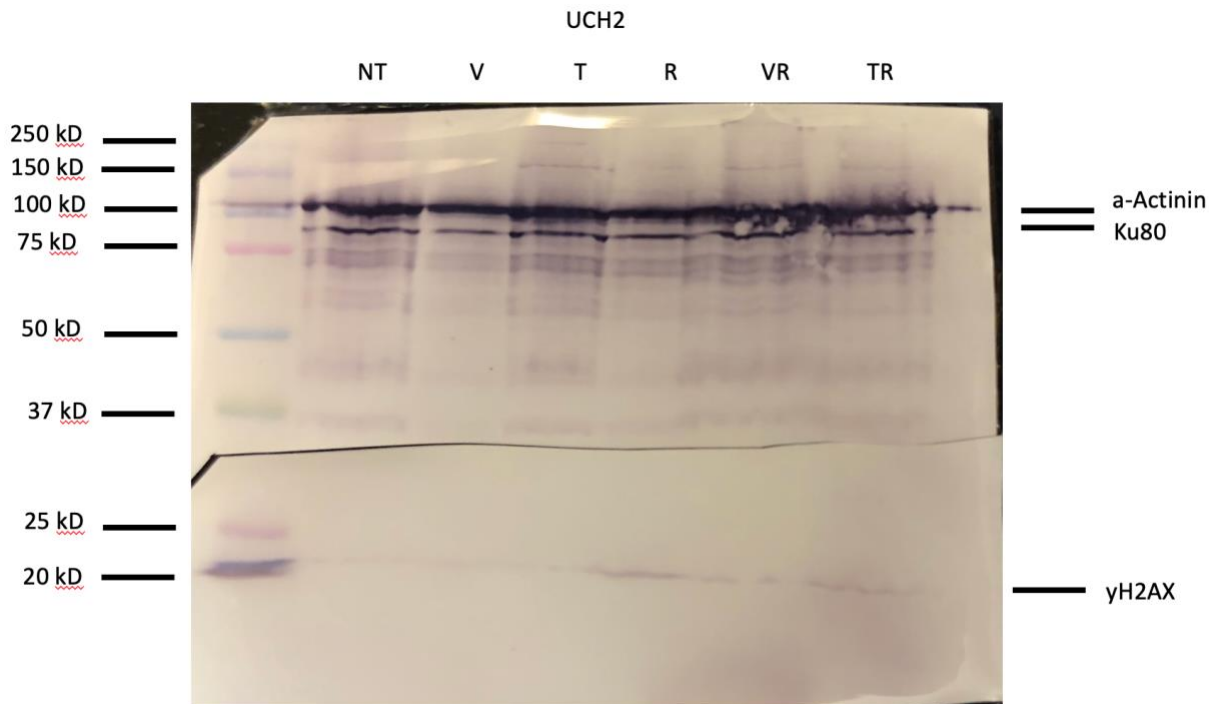


Figure 5. Expression of Ku80 and γ H2AX in the UCH2 cell line. Changes in levels of Ku80 expression are difficult to comment on without band quantification, but it is interesting that γ H2AX, a sensitive marker of double stranded DNA breaks, is seen in the R, VR, and TR groups but not in NT, V, or T. NT= No Treatment, V= Vorinostat, T= Tubacin, R= Radiation (10 Gy), VR= Vorinostat (2.5 μ M) + Radiation (10 Gy), TR= Tubacin (2.5 μ M) + Radiation (10 Gy).

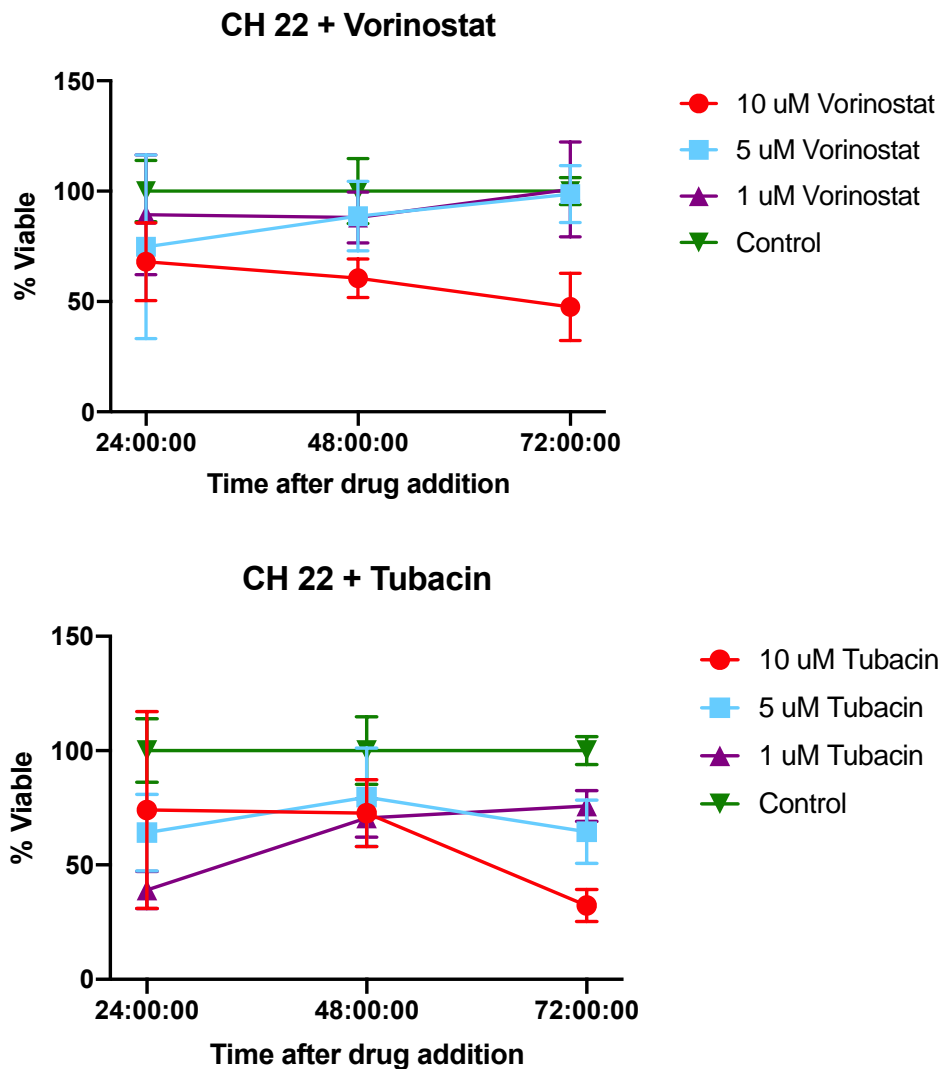


Figure 6. Viability of CH22 after treatment with vorinostat or tubacin. Graphs display mean with standard deviation. CH22 was quite sensitive to 10 uM vorinostat and tubacin at the 72 hr timepoint, but viability remained high at the 1 and 5 uM doses.

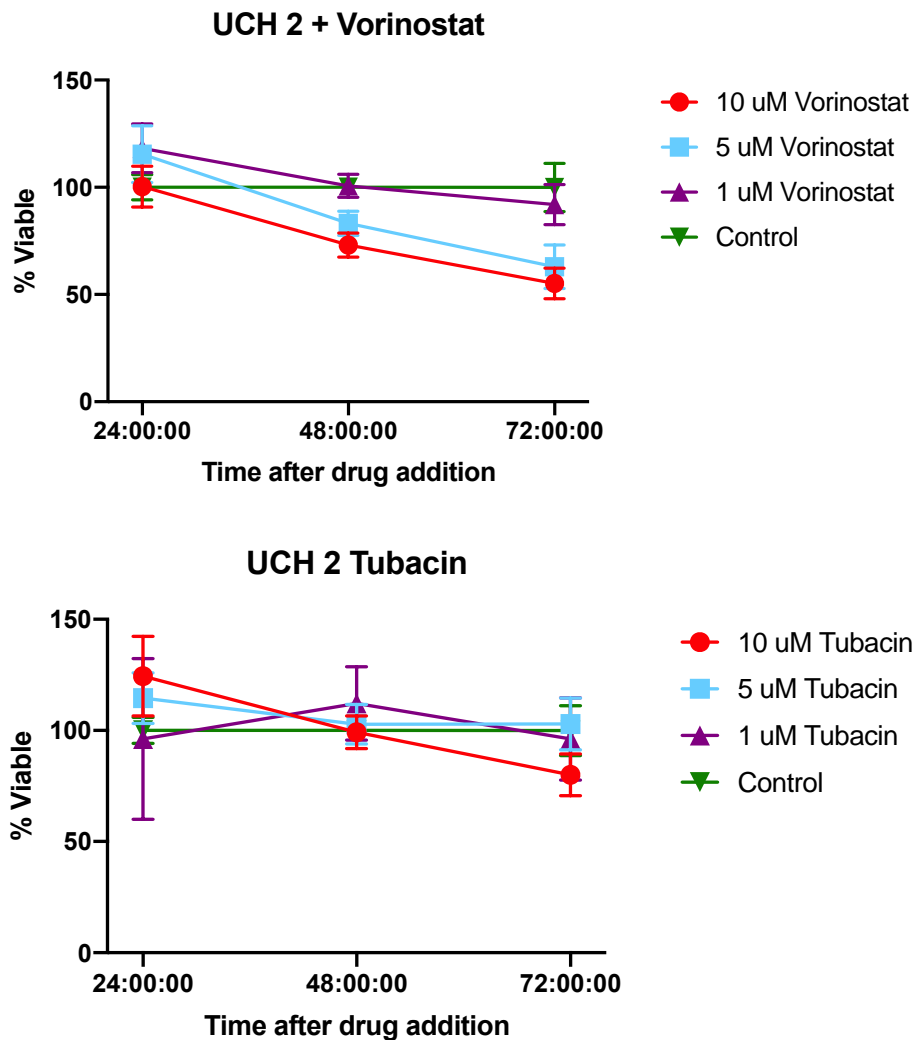


Figure 7. Viability of UCH-2 after treatment with vorinostat or tubacin. Graphs display mean with standard deviation. UCH-2 displayed marked resistance to tubacin at all dose levels, but was somewhat sensitive to the two highest doses of vorinostat.

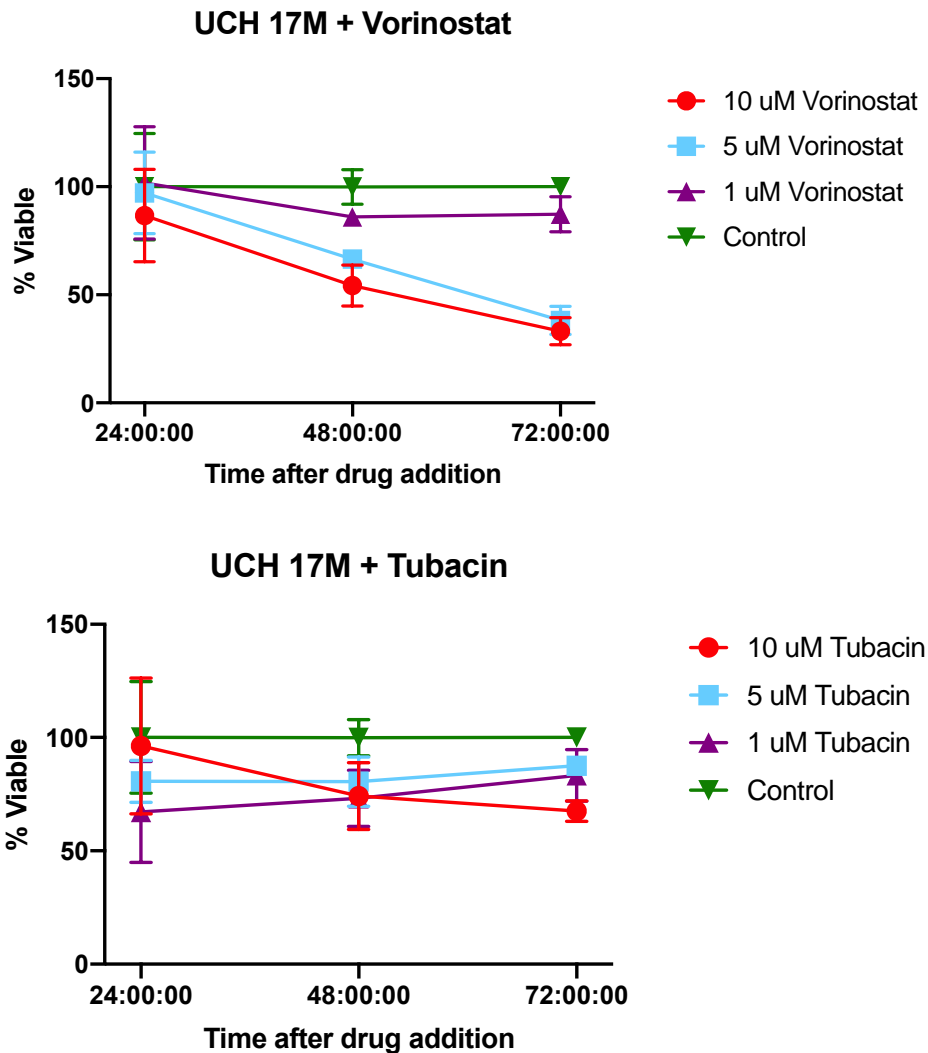


Figure 8. Viability of UCH-17M after treatment with vorinostat or tubacin. Graphs display mean with standard deviation. UCH-17M displayed dose dependent response to vorinostat, but with a significant difference in viability between 1 uM and 5 uM. The two lowest doses of tubacin did not significantly affect viability.

Figures 1-3 illustrate changes in cell viability across all three cell lines following exposure to the HDAC inhibitors vorinostat and tubacin. The purpose of this study was to determine the sensitivity of each of the cell lines to the drug. Given the aim of this project is to

evaluate the effect of combining HDAC inhibition with radiation, it was important that the dose used for future studies did not substantially reduce cell viability when used as a monotherapy. As can be seen, at the 1 uM dosage level both drugs had minimal reduction in cell viability. 5 uM was also generally acceptable, aside from UCH-17M + Vorinostat at 72 hours. Given that doses between 1 and 5 uM for each of the drugs are published and accepted to be low doses, the decision was made to use 2.5 uM as the dose for both drugs moving forward.

To study the combinatorial effects of HDAC inhibition and radiation, cells were then pretreated 24 hours before irradiation with vorinostat or tubacin and then subject to irradiation and subsequent viability testing.

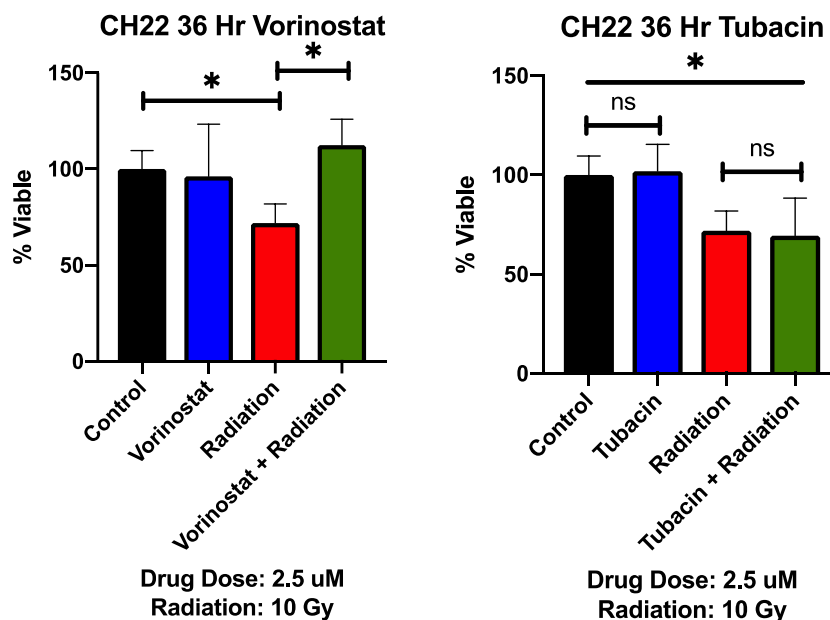


Figure 9. The effects of radiation and HDAC inhibition on CH22 cell viability 36 hours post irradiation. The increased viability seen in vorinostat + radiation group almost certainly originates from a difference in cell number.

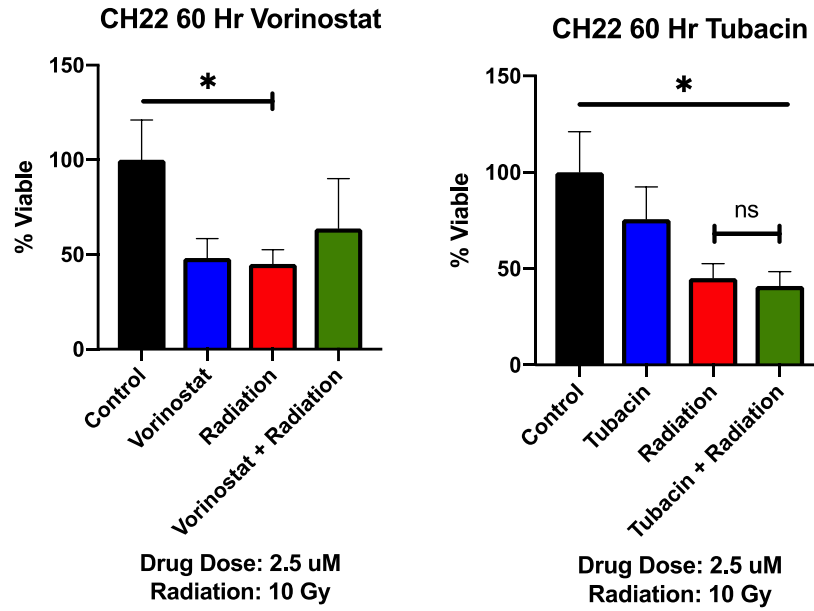


Figure 10. The effects of radiation and HDAC inhibition on CH22 cell viability 60 hours post irradiation. The increased viability seen in vorinostat + radiation group almost certainly originates from a difference in cell number.

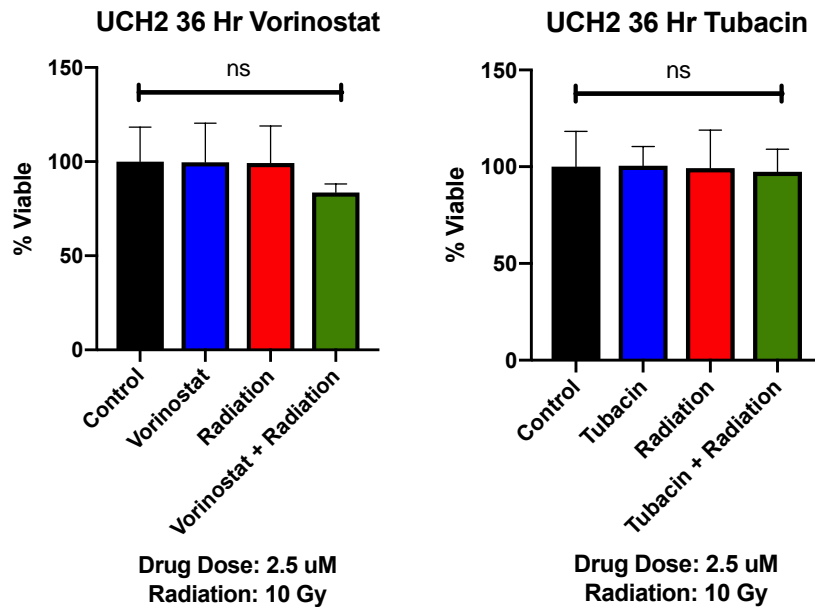


Figure 11. The effects of radiation and HDAC inhibition on UCH2 cell viability 36 hours post irradiation.

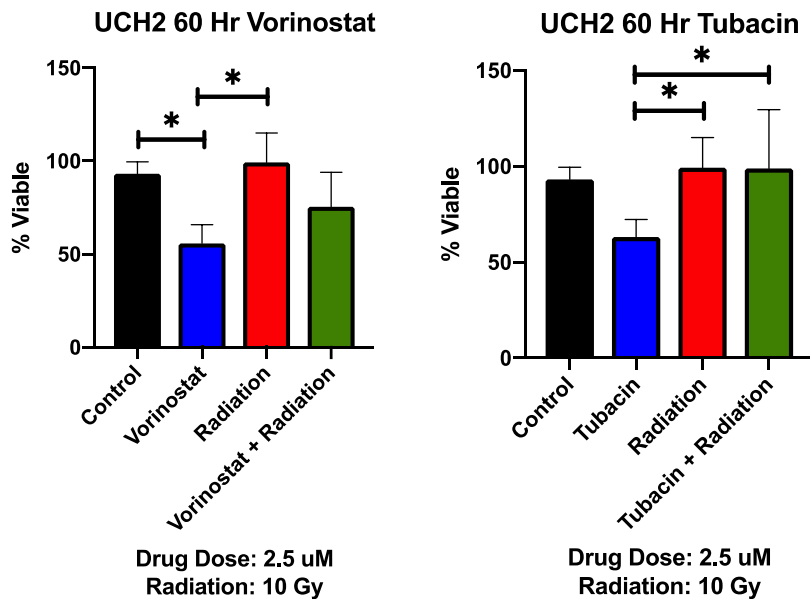


Figure 12. The effects of radiation and HDAC inhibition on UCH2 cell viability 60 hours post irradiation. The increased viability seen in tubacin + radiation group almost certainly originates from a difference in cell number.

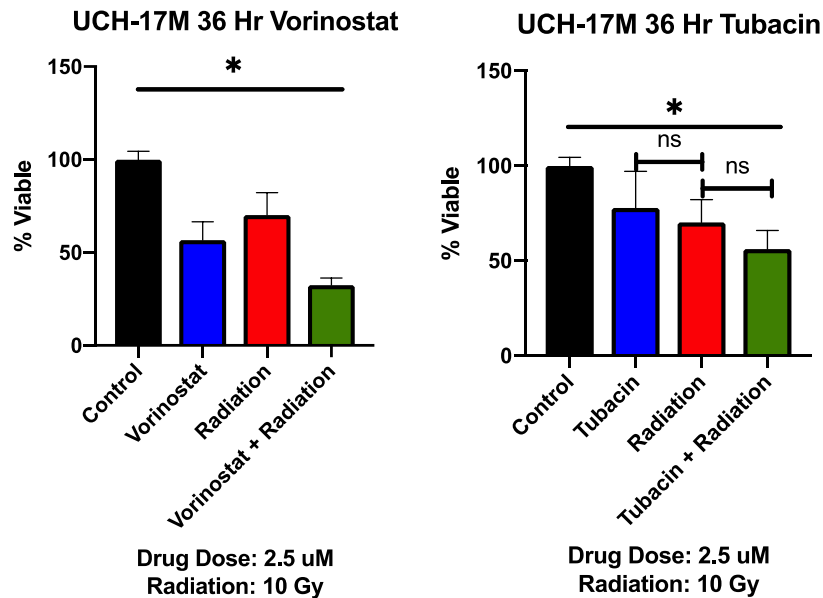


Figure 13. The effects of radiation and HDAC inhibition on UCH-17M cell viability 36 hours post irradiation. Significant radio-sensitization is seen in the vorinostat + radiation group.

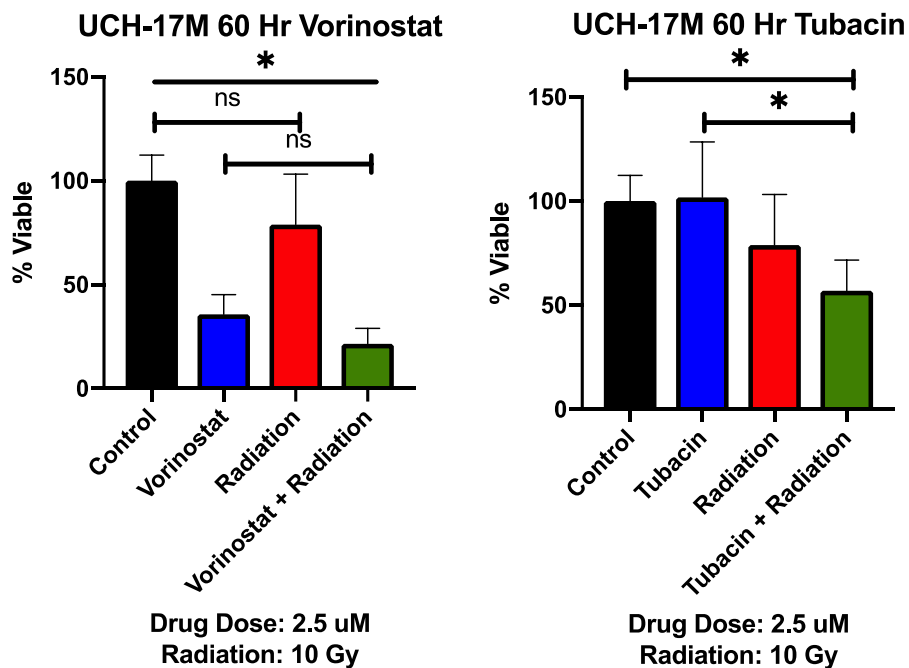


Figure 14. The effects of radiation and HDAC inhibition on UCH-17M cell viability 36 hours post irradiation. Significant radio-sensitization is seen in the vorinostat + radiation group.

Discussion

Western Blots, shown in Figures 2-5, confirm that HDAC 1 and 6 expression seen in the 2013 paper by Schipel *et al.* is also seen in chordoma cell lines. Unfortunately, due to a technical error with the SDS-PAGE gels or the transfer process, blots for UCH-17M could not be developed. As can be seen in Figures 6-8, the effect of monotherapy HDAC inhibition varied between cell lines. CH22 appeared quite sensitive to the highest dose of vorinostat and tubacin, even though these doses are still on the lower end of the ranges seen in the literature. UCH2, on the other hand, appeared quite resistant to both drugs. Finally, UCH-17M was largely resistant tubacin, but was quite sensitive to vorinostat. Ultimately, a dosage of 2.5 uM was chosen for both drugs for subsequent experiments. Because the experiments are internally controlled for drug only cell death, it was possible to ensure that a combinatorial effect could still be seen.

As for the combinatorial effect, only one cell line showed a significant reduction in viability between the radiation and radiation + drug group: UCH-17M and Vorinostat. This evidence suggests that there is some rationale for combining HDAC inhibition with radiation therapy for chordoma. However, it is only fair to present this data in the context of a few serious caveats. Firstly, the cell viability assay used here measures the presence of formazan, generated by the reduction of the MTT dye by NAD(P)H enzymes. Samples with a greater absorbance have more formazan present, indicative of greater NAD(P)H activity. Thus, this assay is sensitive to variation in the number of cells present in each well. While every attempt was made to seed 2000 cells into each well, the workflow used for these experiments generated an unavoidable variable. Because each cell line was divided into 6 groups (NT, Radiation, Vorinostat, Tubacin, Tubacin + Radiation, and Vorinostat + Radiation), these samples had to be seeded independently. Thus, it is possible that some samples had less than 2000 cells per well, and others had more. Given the

assay dependency on cell number, the true results could be skewed by variation in cell number. In theory, a better approach to this assay would be using ThermoFisher Live/Dead stain in combination with CountBright Absolute Counting Beads for flow cytometry. The beads allow for viability to be normalized to cell number, eliminating this error. Unfortunately, because Florida Southern College does not have access to a flow cytometer, it was impossible to use approach.