Understanding the Role of Hydralazine as an Epigenetic Cancer Therapy in Relation to N-Acetyltransferase Acetylator Phenotype

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Introduction

- N-Acetyltransferases (NATs) are acetylating enzymes. NAT1 and NAT2 are isozymes. NAT2 is distributed in the liver and absent in the intestine. NAT1 is predominately found in the intestine.
- Hydralazine is a drug used in resistant hypertension and is subject to acetylation by NAT2. Earlier studies evidenced a tri-modal distribution of acetylation phenotypes (rapid, intermediate, and slow), while more recent, smaller studies demonstrated bimodal (rapid and slow) acetylation phenotypes.
- Recent studies have demonstrated that hydralazine acts as an epigenetic modulator with neoplastic cells, aiding in the phenotypic variation of acetylation of hydralazine with human hepatocyte lysates.
- EARLIEST studies evidenced a tri-modal distribution of acetylation phenotypes (rapid, intermediate, and slow), while more recent, smaller studies demonstrated bimodal (rapid and slow) acetylation phenotypes.

Hypothesis

If hydralazine is shown to be metabolized by NAT1 and NAT2, the study will determine quantifiable differences in the amount of hydralazine available to modulate cellular epigenetics, to be measured in human hepatocytes.

Methods

- Determination of NAT1 vs NAT2 hydralazine enzyme kinetics
  - Yeast lysates of human recombinant NAT1 and NAT2 were used in independent reactions with hydralazine to determine the Km and Vmax. Quantification of the amount of product produced during reactions was determined using HPLC. These reactions established the enzyme kinetics for each isozyme.
- In vitro determination of acetylation rates due to NAT1 genotype
  - Cryopreserved human hepatocytes from rapid, intermediate, and slow genotypes were used in reactions with hydralazine and acetyl coenzyme A. The amount of acetylated product produced during reactions was determined using HPLC. These reactions established the enzyme kinetics for each isozyme.
- Determination of NAT1 vs NAT2 hydralazine enzyme kinetics
  - One Way ANOVA was used in reactions with hydralazine and acetyl coenzyme A. The same experiment was used in reactions with hydralazine and acetyl coenzyme A. The same experiment was used in reactions with hydralazine and acetyl coenzyme A.

Results

- Determination of NAT1 vs NAT2 hydralazine enzyme kinetics
  - NAT2 has a higher apparent Km, signifying increased affinity for hydralazine when compared to NAT1 (Figure 1A). Also, NAT2 has a higher Vmax, resulting in increased production of acetylated hydralazine, when compared to NAT1 (Figure 1B). Thus, NAT2 has a higher substrate clearance of hydralazine (Figure 1C).
- In vitro determination of acetylation rates due to NAT2 genotype
  - At multiple concentrations, the different NAT2 genotypes resulted in quantifiable differences in acetylation rates of hydralazine with rapid acetylators producing the most acetylated product, intermediate acetylators producing intermediate levels, and slow acetylators producing the least. This supports a tri-modal distribution of acetylation phenotypes for hydralazine.

Conclusions

- Determination of NAT1 vs NAT2 acetylation phenotypes
  - The Km and Vmax for hydralazine was determined for NAT1 and NAT2 (n=3). Difference in substrate affinities is significant with respect to NAT2 genotype (p=0.0016). NAT2 has a lower apparent Km, signifying increased affinity for hydralazine, and increased NAT2 acetylation of hydralazine, when compared with NAT1. (Figure 1.B). Thus, NAT2 has a higher substrate clearance of hydralazine than that of NAT1 (p<0.0001).
- In vitro determination of acetylation rates due to NAT2 genotype
  - There was a genotype dependent increase in the amount of acetylated hydralazine produced. Rapid, intermediate, and slow acetylators producing the most, intermediate, and the least amount of acetylated hydralazine respectively at 10 µM hydralazine (p<0.0002) (Figure 4A) and 100 µM hydralazine (p<0.0015) (Figure 4B).

Clinical Significance/ Future Direction

- Clinical significance
  - These results evidence two layers of complexity when finding the appropriate dosing regimen for hydralazine so that maximal epigenetic modulation and phenotypic variation for all acetylating genotypes is achieved.
- Future direction should include quantifying the difference in epigenetic modulation based on NAT2 genotype and specific SNPs for a data driven dosing regimen with equivalent therapeutic efficacy based on a tri-modal hydralazine acetylating index.

Acknowledgements and References


Research supported by a grant from the National Cancer Institute R25-CA134283 and the School of Medicine Summer Research Scholar Program.
Background: The metaphase-anaphase transition is regulated by the spindle assembly checkpoint (SAC). The SAC ensures equal segregation of sister chromatids. The anaphase promoting complex/cyclosome (APCC) is the master regulator of mitosis and is responsible for the metaphase-anaphase transition and licensing DNA replication in early G1. Without a functional APCC, malignant and nonmalignant cell lines arrest in mitosis or G1 state with subsequent cell death. Therefore, in vitro studies were performed to predict hit compounds targeting the APCC. Hypothesis: Hit compounds targeting the APCC will induce mitotic arrest and apoptosis selectively in cancer cells. Methods: AlamarBlue, mitotic indices, and caspase 3/7 assays were performed with hit compounds in non-malignant HBEC3-KT cells and malignant A549 and H460 lung cells to determine the effect of these compounds on proliferation and induction of apoptosis. Photomicroscopy studies were performed prior to the caspase 3/7 assay to determine the optimal conditions for the assay.

Results: AlamarBlue and mitotic index data show hit compounds reduce cell viability and increase mitotic index in all three cell lines. Photomicroscopy and caspase 3/7 assays show selective induction of apoptosis in malignant cells. Discussion: AlamarBlue results do not differentiate between cytotoxic and cytostatic activity. While hit compounds appear to reduce cell viability and increase mitotic index in both malignant and nonmalignant cells, apoptosis assays indicate that only the malignant cells undergo apoptosis in response to mitotic arrest. This suggests that hit compounds are cytostatic, rather than cytotoxic in non-malignant cells.

Conclusions
- Hit compounds arrested both malignant and nonmalignant cells in mitosis with selective induction of apoptosis in malignant cells, consistent with our hypothesis.
- These results suggest these compounds can undergo further structural modifications to candidate drugs that will induce cancer cell death while sparing normal cells.

References

Acknowledgements
Research supported by grants from National Cancer Institute (M01-RR00058, Kentucky Lung Cancer Research Fund (4-03-01)), and Kentucky Science and Engineering Foundation (KSEF-0249-RDE-01B).
### Introduction

- Diagnostic radiology is an important competency that spans multiple medical specialties, and imaging is a necessary tool to evaluate patients in coordination with physical exam.
- However, it is often overlooked during medical education, so it is important to incorporate clinically relevant diagnostic radiology into clerkships during medical students' third and fourth years.
- The purpose of this study is to understand how diagnostic radiology is being taught, what barriers exist to increasing diagnostic radiology education, and possible solutions to those barriers.

### Methods

Data for this project was collected from four sources:

1. A comprehensive review of the literature on diagnostic radiology education during undergraduate medical education, including curriculum, methods of teaching, and outcomes
2. AAMC Curriculum Inventory data on the subject of Radiology education during undergraduate medical education, including online modules, flipped classrooms, and financial constraints
3. A survey of clerkship directors at the University of Louisville in the departments of Emergency Medicine, Internal Medicine, Neurology, OB/GYN, and Surgery
4. The same survey sent to clerkship directors at other institutions

### Results

#### Fig. 1a: Number of Schools Reporting Radiology per Academic Level
- Year 1 has the highest number of schools reporting radiology education.
- The amount of radiology education decreases as the academic level increases.

#### Fig. 1b: Number of Schools Reporting Use of Each Instructional Method in Coverage of Radiology
- The most common methods used are lecture, discussion, and self-assessment.

#### Fig. 1c: Number of Schools Reporting Use of Each Assessment Method to Evaluate Radiology-Related Knowledge
- Exam: independently written,_Computer Assisted Radiology Assessment
- Lab: independently written, hands-on labs, simulation exercises, and externships and triage activities in a clinical setting with radiologists
- Triage: self-assessment, workbooks, and final examination

#### Fig. 2a: Importance of Diagnostic Radiology in Clerkships at the University of Louisville: 4.5 3rd-year and 5/5 4th-year clerkship directors indicate that radiology education is at least moderately important.
- Students, department heads, and medical school deans see value in radiology education.

#### Fig. 2b: Importance of Diagnostic Radiology in Clerkships at Other U.S. Public Medical Institutions: 15/18 3rd-year and 13/18 4th-year clerkship directors indicate that radiology education is at least moderately important.
- Barriers exist to incorporating more radiology education, including time in the curriculum, faculty availability to teach, and financial constraints.

### Conclusions

- There is room for improvement for radiology education in U.S.
- There are examples of initiatives to increase radiology education, including online modules, flipped classrooms, hands-on labs, simulation exercises, and externships and triage activities in a clinical setting with radiologists.
- The future of successful radiology education will be vertical integration into preclinical medical education as well as integration into core clerkships that students are required to take.

### Acknowledgements

Research supported by a grant from the NCI R25 Grant, University of Louisville Cancer Education Program (R25-CA134283) and the School of Medicine Summer Research Scholar Program. I would also like to thank my mentor, Dr. Robert Martin II, MD, PhD for his guidance, and the AAMC for providing curriculum data on Radiology.
IDENTIFYING SERUM EXOSOMAL MICRORNA SIGNATURES AS DIAGNOSTIC TOOLS IN MELANOMA PATIENTS

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ABSTRACT

INTRODUCTION

- Exosomes are 30-120 nm extracellular vesicles that contain mRNA, miRNA, and proteins and are commonly found in body fluids.
- Exosomes are highly involved in cell-cell communication via autocrine and paracrine signaling.
- MicroRNAs (miRNAs) are small, single-stranded, noncoding RNA molecules that post-transcriptionally alter mRNA and regulate gene expression.
- Some of the miRNAs found in tumor exosomes have been shown to contribute significantly to the progression of malignant melanoma.
- Analyzing exosomal miRNA signatures in melanoma patients could identify a potential biomarker for early diagnostic assessment and prognosis.

METHODS

1. Exosomes were isolated from patient serum using two separate isolation protocols - ExoRNeasy (Dataset 1) and ExoQuick (Dataset 2).
2. RNA was extracted from exosomes.
3. miRNAs were selected from previous experiments by Affymetrix miRNA array 2.0.
4. RT-PCR was performed to analyze and confirm differential expression of specific exosomal miRNAs among patient subgroups in Dataset 1 and Dataset 2.

RESULTS

- Table 2: Fold changes of differentially expressed miRNAs in Stage IV vs. non-melanoma patients
- Table 3: Fold changes of differentially expressed miRNAs in Stage IV vs. non-melanoma patients
- Table 4: Fold changes of differentially expressed miRNAs in Stage IV vs. Stage I non-melanoma patients

CONCLUSIONS

- Specific exosomal miRNAs were found to be differentially expressed as a function of melanoma disease status - many of which were consistently downregulated.
- These differences in miRNA expression allow for alterations in gene regulation that may be targeted to play a role in the development and progression of malignant melanoma.
- Our results provide evidence that further characterization of exosomal miRNA signatures in melanoma patients of varying disease stages is warranted.
- This research could eventually lead to the development of a minimally invasive method for specific and early diagnosis of malignant melanomas.

ACKNOWLEDGEMENTS

Research supported by NCI R25 grant University of Louisville Cancer Education Program NIH/NCI (R25 CA143828), grant from Melanoma Research Foundation, and University of Louisville Clinical & Translational Science Pilot Grant Innovative Award to K.M.M.
UBQLN1 Regulates Expression of EGFR\textsuperscript{WT} but not EGFR\textsuperscript{mut} in Lung Adenocarcinoma Cells

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Role of EGFR in Tumorigenesis

Abstract

Introduction: Lung cancer is the second most common cancer in men and women and the leading cause of cancer-related deaths worldwide. Adenocarcinomas represent approximately 40% of all lung cancer cases. Epidermal Growth Factor Receptor (EGFR) is a tyrosine kinase receptor involved in proliferation pathways. Mutations in the EGFR gene, a known oncogene, account for 10-15% of adenocarcinoma cases. Of the known mutations, some make adenocarcinomas more sensitive to treatment with tyrosine kinase inhibitors (TKIs), while other mutations increase resistance to TKIs. The Ubiquilin family is a family of proteins that have ubiquitin-like functions and are also involved in regulation of proteins like anti-apoptotic BCL\textsubscript{b} and another receptor tyrosine kinase, IGF1R. UBQLN1 function is lost in 50% of lung adenocarcinoma cases. The Beverly lab has discovered an interaction between EGFR and UBQLN1. We aim to study regulation of EGFR by UBQLN1 and implications of this association in lung cancer progression.

Objective: We hypothesize that UBQLN1 regulates EGFR expression and activity, and that loss of UBQLN1 makes lung adenocarcinoma cells more tumorigenic.

Methods: We used two lung adenocarcinoma cell lines in our studies: A549 (EGFR\textsuperscript{WT}, K\textsuperscript{-}RAS\textsuperscript{G12S}) and H1650 (EGFR\textsuperscript{A746del750}, K\textsuperscript{-}RAS\textsuperscript{WT}). We studied regulation of EGFR by UBQLN1 using the following methods: Immunoprecipitation, Western Blot analysis, Alamar Blue assay, and qRT-PCR.

Results: We found that following loss of UBQLN1 in A549 cells, there is decreased expression of EGF receptors when stimulated with EGF compared to wild-type. However, the ratio of phosphorylated to total EGFR is higher in cells with loss of UBQLN1 function. In H1650 cells, we did not find such a difference.

Conclusion: We conclude that UBQLN1 is critical in regulation of wild-type EGFR but not constitutively active EGFR. Loss of UBQLN1 leads to persistent stimulation of EGFR which may contribute to tumorigenic events in UBQLN1 deficient cells that have wild-type EGFR. Therefore, this receptor could be an appropriate target in EGFR\textsuperscript{WT} cancers that have loss of UBQLN1 function.

Hypothesis

We hypothesize that UBQLN1 regulates EGFR expression and activity, and that loss of UBQLN1 makes lung adenocarcinoma cells more tumorigenic.

Future Directions

• Look at number of EGF receptors in the presence of Bortezomib, a proteasomal inhibitor
• Repeat degradation kinetics experiment in the presence of Cyclohexamide, an inhibitor of de novo protein synthesis
• Check for receptor saturation at higher dose of EGF

Acknowledgements

Research was supported by the NCI R25-CA134283 Cancer Education Program grant, the NCI R01-CA193220 grant, and the James Graham Brown Cancer Center.
IDENTIFICATION OF THE ENDOGENOUS ROLE OF ARYLAMINE N-ACETYLTRANSFERASE 1 IN CANCER RELATED CELLULAR PROCESSES THROUGH PROTEOMIC ANALYSIS

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ABSTRACT

Background: Arylamine N-acetyltransferase 1 (NAT1) is a cytosolic enzyme that catalyzes the transfer of acetyl groups to xenobiotics and endogenous compounds. Knockout studies have shown that the absence of NAT1 leads to increased toxicity. Many of these studies have used cell lines with variable NAT1 expression and thus, the endogenous role of NAT1 is still not fully understood.

Methods: Various cell lines were exposed to arginine and aminoguanidine. The expression levels of NAT1 were determined via western blotting. The cell lines were then subjected to a comparative proteomics analysis using both control and NAT1 knockout cell lines.

Results: From this analysis, we identified various proteins that were significantly affected in both cell lines. In particular, we identified 4890 proteins with a false discovery rate of 3%. Among these proteins, we found that oxidative phosphorylation, apoptosis, and survival were significantly increased in the #5 knockout.

Discussion: Our results suggest that NAT1 has a significant role in the regulation of these processes. Further studies are needed to elucidate the underlying mechanisms and to better understand the role of NAT1 in cancer and toxicity.

RESULTS

Figure 1: This figure shows the top 10 significantly affected pathways in the #5 knockout cell line. The pathways are ranked based on their Log2 fold change.

Figure 2: This figure shows the top 10 significantly affected pathways in the parental cell line. The pathways are ranked based on their Log2 fold change.

Figure 3: This figure shows the top 10 significantly affected pathways in both cell lines. The pathways are ranked based on their Log2 fold change.

Table 1: This table lists the top 10 significantly affected pathways in both cell lines. The pathways are ranked based on their Log2 fold change.

HYPOTHESIS

We hypothesized that a comparative proteomics analysis of parental versus NAT1 knockout cell lines would identify differences in protein expression that are associated with varying NAT1 expression between the cell lines. We predicted that oxidative phosphorylation, apoptosis, and survival would be more highly expressed in the cell lines with higher NAT1 expression.

METHODS

Cell lines: Two cell lines were used in this study: parental and NAT1 knockout cell lines.

Quantitative Proteomics: Quantitative Proteomics using Liquid Chromatography-Mass Spectrometry (LC-MS/MS) was used to acquire global protein levels in 3 samples of each cell type. The samples were processed in triplicate and the results were normalized to the average of the 9 samples. This yielded 4890 proteins with a false discovery rate of 3%.

RESULTS

Table 1: This table lists the top 10 significantly affected pathways in both cell lines. The pathways are ranked based on their Log2 fold change.

DISCUSSION

Pathway analysis generated through MetaCore software yielded interesting potential pathways affected by enzymatic NAT1 activity. Among these, oxidative phosphorylation, apoptosis, and survival were the most consistently affected across both cell lines with significant changes in protein expression.

FUTURE DIRECTION

These findings provide the groundwork for a mechanistic approach to NAT1 enzymatic activity and can be used to further elucidate the role of NAT1 in cancer and toxicity.