The Relationship Between NAT1 and *N*-Acetylasparagine or *N*-Acetylputrescine

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Abstract

•Human arylamine *N*-acetyltransferase 1 (NAT1) is a phase II xenobiotic metabolizing enzyme. High levels of NAT1 have been associated with luminal and estrogen receptor positive breast cancers (BCs), knockout of NAT1 causing decreased anchorage-dependent growth and invasive ability of BC cells. Our lab has demonstrated a statistically-significant association between NAT1 *N*-acetylation and the levels of *N*-acetylasparagine and *N* -acetylputrescine in MDA-MB-231 BC cells. High levels of Asparagine (ASN) have been shown to increase the metastasis and invasive ability of BC cells, while inhibition of a putrescine (PUT) biosynthesizing enzyme has shown decreased BC cell proliferation; acetylation of ASN and PUT is one potential regulatory mechanism governing bioavailability of ASN and PUT. Given the statistical correlation between NAT1 activity levels and *N*-acetylasparagine and *N*-acetylputrescine, we hypothesize that NAT1 acetylates ASN or PUT. To determine if either ASN or PUT are substrates of NAT1 *N*-acetylation, *in vitro* competition assays between ASN or PUT and known NAT1 substrate *para*-aminobenzoic acid (PABA) were conducted. *In vitro* NAT1 ASN competition showed no changes in *N*-acetylated PABA (AcPABA) levels in response to ASN. *In vitro* NAT1 PUT competition showed decreased AcPABA levels giving an IC_{50} value of 2.50mM. To determine if NAT1 responds similarly in BC cells, we also conducted experiments to test whether PUT inhibited NAT1 *N*-acetylation of PABA in MDA-MB-231 cells; there was 10% inhibition at 5mM PUT. To elucidate if PUT was inhibiting NAT1 *N*-acetylation of PABA by competitive inhibition, *in vitro* kinetic assays were conducted; the data supports PUT as an uncompetitive inhibitor of NAT1 PABA *N*-acetylation. In conclusion, *in vitro* data support that PUT is a NAT1 substrate both in yeast following recombinant expression of NAT1 and in MDA-MB-231 adenocarcinoma BC cells; *In vitro* data for ASN competition did not show ASN competing with PABA as a NAT1 substrate. To validate these results, our lab is currently developing a high pressure liquid chromatography mass spectrometry (HPLC-MS) method to measure *N*-acetylasparagine and *N*-acetylputrescine. In future studies to determine the impacts of ASN or PUT and NAT1 in BC cells, MDA-MB-231 BC cells expressing varying levels of NAT1 will be exposed to varying levels of ASN or PUT and investigated for changes in their behaviors.

Methods

•***In vitro* NAT1 *N*-acetylation activity assays to determine competition between ASN and PABA** were conducted using human NAT1-expressing yeast lysates. Competition assays utilized fixed PABA and AcCoA and ASN concentrations from 0.622mM-3mM. Reactions were stopped after 10 minutes. Amounts of AcPABA were measured via high pressure liquid chromatography (HPLC) analysis.

•***In vitro* NAT1 *N*-acetylation activity assays to determine competition between PUT and PABA** were conducted using human NAT1-expressing yeast lysates. Competition assays utilized fixed PABA and AcCoA and PUT concentrations from 1.37mM-10mM. Reactions were stopped after 11min. Amounts of AcPABA were measured as above.

•***In situ* NAT1 *N*-acetylation activity assays** were conducted in MDA-MB-231 human cells that endogenously express NAT1. The cells were incubated in media supplemented with 5mM, 2.5mM, and 1.25mM PUT and PABA. Media was collected at 48hr time points, amounts of AcPABA were measured as above.

•***In vitro* assays to determine PUT inhibition mode of NAT1 *N*-acetylation activity** were conducted using human NAT1-expressing yeast lysates. Assays utilized PABA concentrations from 250uM-4mM and PUT concentrations of IC_{50} or IC_{75}. Amounts of AcPABA product were measured as above.

•***In vitro* assays to determine NAT1 *N*-acetylation of PUT or ASN** were conducted using human NAT1-expressing yeast lysates. Reactions used 300uM PABA, 1mM AcCoA, and 300uM ASN or 300uM PUT; CoA levels were measured at 0min, 10min, and 60min. Acetylation of PUT or ASN was determined via measurement of CoA levels using BioVision (Milpitas, CA) fluorometric Co-enzyme A (CoA) assay kit.

Hypotheses

•**NAT1 is acetylating ASN** to produce *N*-acetylasparagine. Competition between ASN acetylation and PABA acetylation will lead to decreased AcPABA levels in the presence of ASN.

•**NAT1 is acetylating PUT** to produce *N*-acetylputrescine. Competition between PABA acetylation and PUT acetylation will lead to decreased AcPABA levels in the presence of PUT.

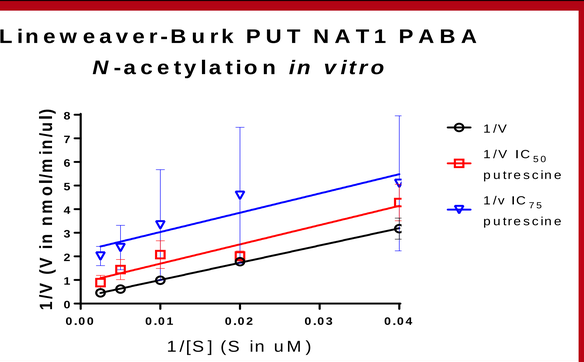
1

L-asparagine N-acetylasparagine

2

Putrescine N-acetylputrescine

Results



•**Figure 2. Determination of mode of inhibition of PUT** on NAT1 PABA *N*-acetylation. Lineweaver-Burk plot of the reciprocal of the velocity of AcPABA production (1/V) over the reciprocal PABA concentrations (1/[S]). Reactions were conducted *in vitro* in yeast expressing human recombinant NAT1. PABA concentrations ranged 25uM to 400uM and PUT concentrations of IC_{50} or IC_{75} *in vitro*. Decreased K_m and V_{max} observed in inhibited reactions supports PUT as a uncompetitive inhibitor. Replicates were conducted (n=3).

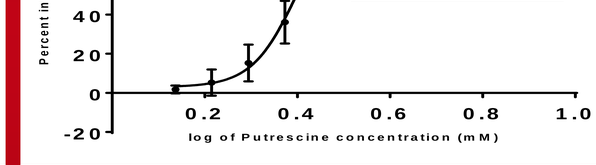


Figure 3. *In vitro* NAT1 PABA *N*-acetylation activity in presence of PUT. Percent inhibition was determined by use of 1mM AcCoA and 300uM PABA with 1.37mM-10mM PUT using yeast expressing human recombinant NAT1. PUT caused a dose-dependent decrease of AcPABA production in the recombinant yeast. The calculated IC_{50} for inhibition was 2.50 ± 0.10mM. Replicates were conducted (n=3).

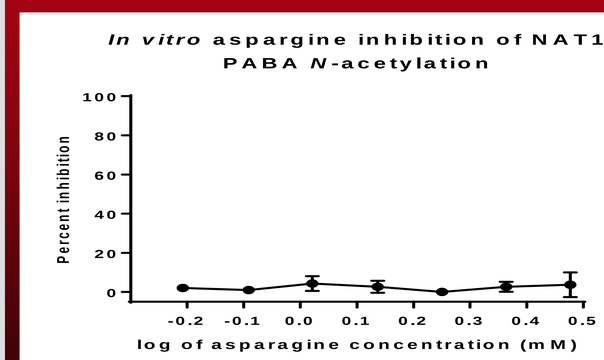
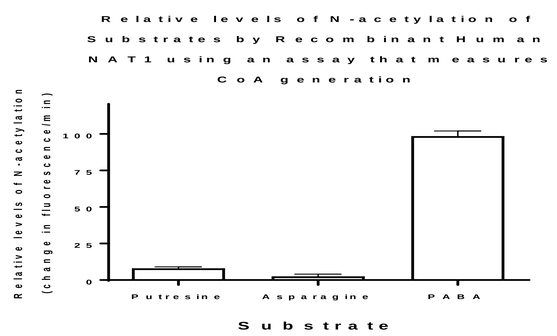
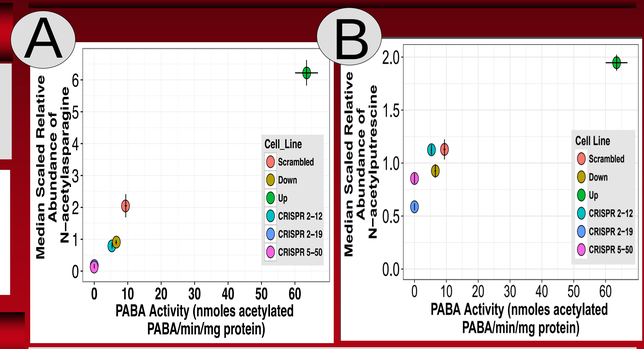


Figure 4. *In vitro* NAT1 PABA *N*-acetylation activity in presence of ASN. Percent inhibition was determined by use of 1mM AcCoA and 50uM PABA with ASN concentrations from 0.622mM-3mM using yeast lysate expressing human recombinant NAT1. Replicates were conducted (n=3).



•**Figure 5. *In vitro* determination of NAT1 *N*-acetylation of ASN or PUT.** Levels of *N*-acetylation of PUT, ASN, and PABA were determined using BioVision (Milpitas, CA) fluorometric CoA assay kit to measure CoA levels. CoA levels were measured at 0min, 10min, and 60min. Reactions were conducted using 300uM PABA, 1mM AcCoA and 300uM ASN or 300uM PUT in yeast expressing human recombinant NAT1. Reactions were stopped at 60min. Replicates were conducted (n=3).

Introduction



•**Figure 1. statistically-significant correlation between NAT1 activity levels and N-acetylasparagine (A) and N-acetylputrescine (B) levels**, as measured in MDA-MB-231 breast cancer cells. Short-hairpin RNA (scrambled, Down, and Up) or CRISPR/CAS9 technology (CRISPR 2-12, 2-19, and 5-50) was used to create MDA-MB-231 cell lines that expressed varying levels of NAT1. Reprinted from Carlisle et al. (2018) Untargeted Metabolomics of Transformed MDA-MB-231 Breast Cancer Cells Expressing Varying Levels of Human Arylamine *N*-Acetyltransferase 1 (NAT1) Suggests a Role for NAT1 in Amino Acid, Lipid, and Fatty Acid Metabolism. *In preparation.*

•**Human *N*-acetyltransferase 1** (NAT1) is a phase II xenobiotic metabolizing enzyme that uses acetyl-coenzyme A (AcCoA) to *N*-acetylate various substrates.

•High levels of NAT1 have been associated with luminal breast cancer (BC) cells and estrogen receptor positive breast cancers.

•**Asparagine (ASN),** a nonessential amino acid, has shown to increase metastasis and proliferation of BC cells in high concentrations.

•**Putrescine (PUT)**, a polyamine synthesized in living cells, has been shown to be involved in BC cell proliferation.

•Our lab has shown a statistically-significant correlation between NAT1 activity and levels of *N*-acetylasparagine and *N*-acetylputrescine in MDA-MB-231 triple-negative adenocarcinoma BC cells, which endogenously express NAT1.

•**The purpose of this study is to determine the relationship between ASN or PUT in NAT1 *N*-acetylation activity.**

Conclusions

- **ASN is not a preferred substrate for NAT1** *in vitro* when compared to PABA.
- **PUT inhibits NAT1 acetylation of PABA *in vitro* and *in situ***, generating IC_{50} value of 2.50mM *in vitro* and showing 10% inhibition at 5mM PUT *in situ*. Attempts to treat *in situ* with larger PUT concentrations resulted in cell death.
- **PUT is an uncompetitive inhibitor for NAT1 PABA *N*-acetylation**.
- **NAT1 is indicated to acetylate ASN and PUT *in vitro*** with increasing reaction times.
- **Future directions** will include verifying NAT1 acetylation of ASN or PUT via HPLC-MS and determining the impact of NAT1 and PUT levels on characteristics of MBA-MB-231 BC cell line.

Acknowledgements

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