Metabolomics of anacardic acid-treated triple negative breast cancer cells

Claire Poulton[1], Kellianne M. Piell[1], David J. Schultz[2], and Carolyn M. Klinge[1]

[1]Department of Biochemistry & Molecular Genetics, University of Louisville School of Medicine

[2]Department of Biology, University of Louisville, Louisville, KY 40292

ABSTRACT

Breast Cancer (BC) is the second leading cause of cancer death in women in the U.S. for 2021. BC is a heterogeneous disease defined by expression of three receptors: estrogen receptor (ER), progesterone receptor, and Human Epidermal Growth Factor 2 (HER2/ERBB2). Triple Negative Breast Cancer (TNBC) lacks expression of all three, meaning it can only be targeted by chemotherapy, and patients have lower overall survival compared to patients whose tumors express ER or HER2. TNBC is more common in Black, Latinx, and younger women. Cancer stem cells (CSCs) within TNBC tumors are resistant to chemotherapy and lead to metastasis. Previous work showed that a natural compound called anacardic acid (AnAc) inhibited the proliferation of TNBC cells and reduced the abundance of the stearoyl-CoA desaturase (SCD) mRNA in MDA-MB-231 TNBC cells. SCD is responsible for the synthesis of Monounsaturated Fatty Acids (MUFAs) that lead to the production of CSCs. TNBC shows altered cellular metabolism compared to ER+ breast tumors, so an unbiased metabolomic analysis can identify new potential targets for TNBC therapeutics based on cancer metabolic pathways. How AnAc affects the cellular metabolome in TNBC, the expression of stearoyl-CoA desaturase (SCD) and other genes regulating lipid synthesis, and CSC in TNBC is unknown and were examined here. MetaboAnalyst v5.0 analysis of the global metabolome identified key metabolic differences between control and AnAc-treated TNBC cell lines and MCF-7 ER+ BC cells. The top 25 AnAc-regulated metabolites identified were associated with metabolic pathways including alanine, aspartate, and glutamate metabolism, glutathione metabolism, and the citric acid (TCA or Kreb's) cycle. Western blots revealed that AnAc downregulated SCD protein in both MDA-MB-231 and MDA-MB-468 and concordantly decreased levels of palmitoleic and oleic acids. On the other hand, HCC1806 TNBC cells showed increased SCD protein and increased oleic acid, but not palmitoleic acid. Further analysis of regulation of SCD is needed to understand this result. Flow cytometry analysis shows a decreasing trend of CSCs with AnAc treatment in MCF-7 and HCC1806 cells, but further experiments are needed to evaluate this finding.

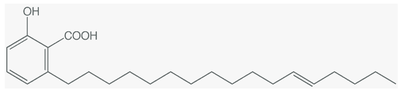
OBJECTIVES

- Identify AnAc-regulated metabolites that may be targets for TNBC therapeutics
- Determine if AnAc prevents the growth of TNBC cells by reducing the SCD enzyme in the monounsaturated fatty acid (MUFA) synthesis pathway, thus reducing TNBC cancer stem cells

BACKGROUND

Cashew Apple

In its natural form, **Anacardic Acid** is found in a popular South and Central American beverage, cashew apple juice, and in mangos.



- Purified anacardic acid (AnAc 24:1$^{\omega5}$) inhibited the proliferation of ERα-positive and TNBC breast cancer cells, but not primary normal human mammary epithelial cells (HMECs) or MCF-10A breast epithelial cells.
- Transcriptome analysis of AnAc-treated MCF-7 and MDA-MB-231 cells revealed that AnAc inhibited SCD and stimulated endoplasmic reticulum stress in MDA-MB-231 TNBC cells.

MATERIALS AND METHODS

Materials: AnAc 24:1$^{\omega5}$ was purified to greater than 95% as previously reported (Schultz *et al*. Mol. Can. Res. 9:594-610, 2010). AnAc was dissolved in ethanol (EtOH); thus, EtOH was used as a vehicle control.

Cell lines: MCF-7, MDA-MB-231, MDA-MB-468, HCC1806, and BT20 breast cancer cells were purchased from American Type Tissue Collection (ATCC, Manassas, VA). All cell lines were verified by short tandem repeat (STR) genotyping (Genetica, LabCorp, Burlington, NC, USA).

Treatments: Cells were grown in phenol red-free IMEM (ThermoFisher) medium containing 5% dextran coated charcoal (DCC)-stripped FBS (hormone-depleted medium) for 48h prior to treatment with AnAc 24:1$^{\omega5}$.

Metabolomics: Metabolites were identified by GC/MS-based metabolite profiling against standards by West Coast Metabolomics Center (U.C. Davis) as described in (Fiehn *et al*. Plant J. 53: 691-704, 2008). MetaboAnalyst 5.0 (Pang *et al*. Nucleic Acids Res. 49: W388-96, 2021) and MetaCore ver. 21.1 (Cortellis, Clarivate, Philadelphia, PA) were used for data analysis and interpretation.

Western blots: Whole cell extracts (WCE) were prepared, separated on 10% SDS-PAGE, transferred to PVDF membranes (BioRad) and immunoblotted with antibodies: SCD (Protein Tech #23393), FASN (BD Biosciences #610963), α-tubulin (ThermoFisher Scientific #MS-81-P1). Blots were stained with Ponceau S for additional quantification. Blots were imaged in a Bio-Rad ChemiDoc™ XRS+ System with Image Lab™ Software.'

Fluorescent-activated cell sorting (FACs) for cancer stem cell (CSC) makers: Cells were stained with fluorochrome-conjugated monoclonal antibodies against human CD44 (mouse allophycocyanin (APC) anti-CD44 antibody cat# 559942; BD Biosciences) and human CD24 (mouse PE anti-CD24 antibody cat# 555428; BD Biosciences) and analyzed using BD LSRFortessa™ Flow Cytometer (BD Biosciences). For each cell line, gating was performed on unstained cells, and the percentages of CD44^{+}/CD24$^{-/low}$ cells were calculated based on the gated unstained control populations.

Table 1: Breast cancer cell lines.

Cell line	Breast cancer patient and tumor characteristics	AnAc Cell viability 48h IC_{50} μM	μM AnAc 24h for metabolomics
MCF-7 ATCC	ERα+/PR+/HER2- luminal A, WT P53; PIK3CA E545K	29	29
BT-20	white, TNBC: Basal A, p53 mut, BRCA WT, PI3CAmut PIK3CA H1047R	40	40
HCC1806	Black, TNBC: Basal-like BL2, p53 mut, BRCA WT,	27	27
MDA-MB-231	white, TNBC: Basal B, p53 mut, BRCA WT, WT PI3K; KRAS G13D, claudin-low	21	20
MDA-MB-468	Black, TNBC: Basal A, p53 mut, BRCA WT, PTEN homo deletion	30	20

RESULTS

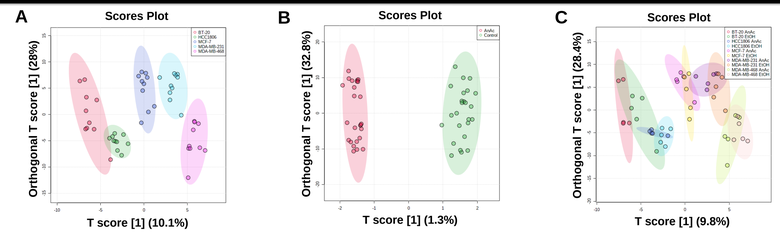


Figure 1: MetaboAnalyst chemometrics analysis using orthoPLS-DA. Each point is representative of an analyzed sample. (A) Statistical analysis shows the distinct characteristics of each TNBC cell line. (B) Further analysis shows robust separation between AnAc and EtOH control samples, independent of cell line type. (C) Separation that occurs when samples are organized by both cell line and treatment.

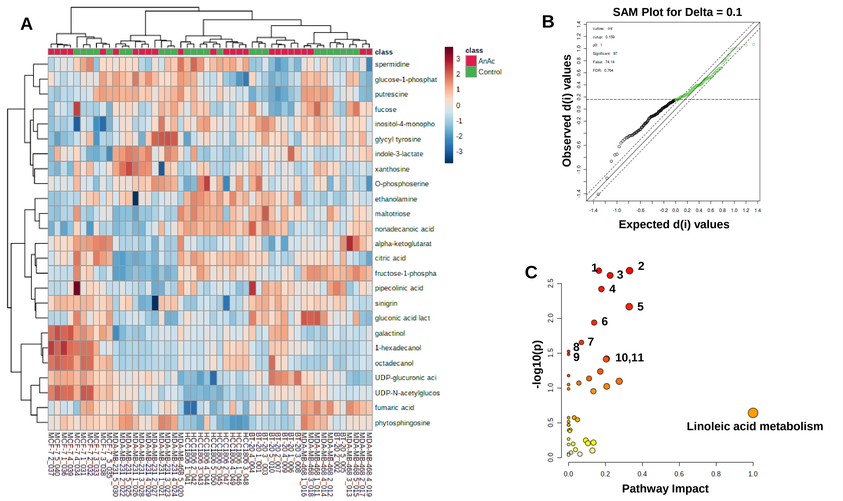


Figure 2: Hierarchical clustering of top 25 metabolites, Significance Analysis of Metabolites (SAM), and Pathway Analysis. (A) Heatmap showing metabolites in cell lines with control (EtOH) or AnAc treatment from ANOVA analysis. (B) SAM shows that 97 metabolites of the dataset were significant out of all cell lines, which these metabolites were then used in pathway analysis (C) to determine the most impacted metabolic pathways. The dark red circles are the most significant.

Table 2: Summary of SAM results for AnAc-altered metabolites and AnAc-regulated pathways.

Top 10 Metabolites	Top Pathways (p < 0.05)	Metabolites Involved in Pathway
fructose-1-phosphate	1. Alanine, aspartate and glutamate metabolism	L-alanine, citric acid, pyruvate, 2-oxoglutarate, L-glutamine, fumarate
sinigrin	2. Glutathione metabolism	putrescine, spermidine, spermine, glutathione, cys-gly, L-cysteine
putrescine	3. Citrate cycle (TCA cycle)	pyruvate, phosphoenolpyruvate, fumarate, 2-oxoglutarate, citric acid
citric acid	4. Pentose phosphate pathway	gluconic lactone, D-gluconic acid, D-ribulose-5-phosphate, D-ribose, D-glycerate
fucose	5. Lysine degradation	L-lysine, saccharopine, L-2-aminoadipate, 2-oxoadipate, L-pipecolate
gluconic acid lactone	6. Pyrimidine metabolism	L-glutamine, CMP, cytidine, uridine, uracil, thymine
glucose-1-phosphate	7. Glycine, serine and threonine metabolism	D-glycerate, O-phospho-L-serine, pyruvate, L-cystathionine, L-cysteine
maltotriose	8. Arginine biosynthesis	2-oxoglutarate, L-glutamine, fumarate
inositol-4-monophosphate	9. D-Glutamine and D-glutamate metabolism	L-glutamine, 2-oxoglutarate
ethanolamine	10. Glycolysis / Gluconeogenesis	D-glucose-1-phosphate, beta-D-glucose, phosphoenolpyruvate, pyruvate
	11. Arginine and proline metabolism	putrescine, spermidine, spermine, hydroxyproline, pyruvate

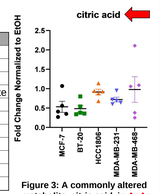


Figure 3: A commonly altered metabolite, citric acid, in AnAc-treated cells relative to EtOH.

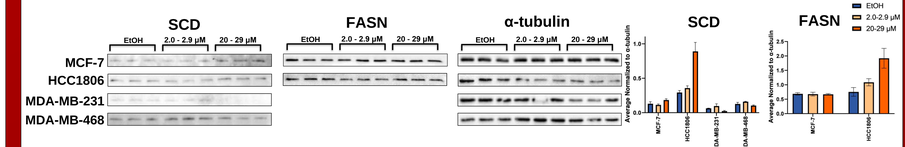


Figure 4: SCD and FASN protein expression after AnAc treatment for 24 h. Western blot analysis showed increased SCD expression in MCF-7 and HCC1806 cell lines and decreased SCD expression in MDA-MB-231 and MDA-MB-468 cell lines relative to α-tubulin. Samples were tested in triplicate, starting with EtOH treated vehicle control followed by 2 increasing concentrations of AnAc treatment based on IC_{50} values (Table 1). FASN expression remained unaffected in MCF-7 samples while increasing in HCC1806 samples.

Table 3: Expected change in metabolites based on SCD expression in western blots.

Expected Metabolite Change:				
SCD expression	palmitic acid	stearic acid	palmitoleic acid	oleic acid
increased	↓	↓	↑	↑
decreased	↑	↑	↓	↓

Agrees with hypothesis of SCD regulation

Disagrees with hypothesis of SCD regulation

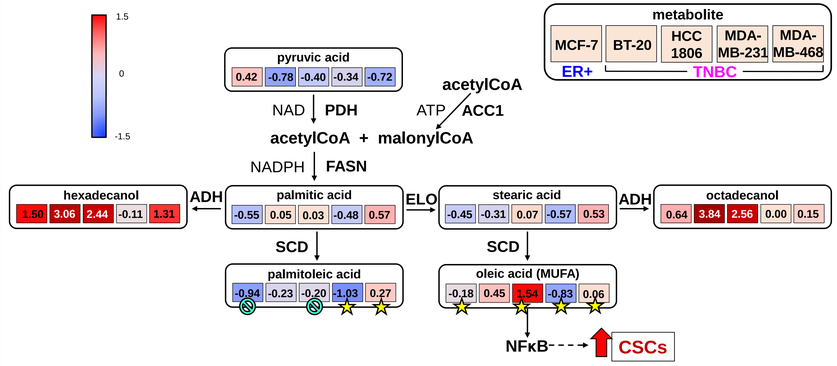


Figure 5: CSC synthesis pathway of metabolites involving SCD and FASN expression. Data are avg. log fold change of AnAc treated samples relative to the EtOH control samples (n = 5). Two additional alcohol metabolites, hexadecanol and octadecanol, are included as they show significant changes in TNBC lines. Their correlation to SCD is unknown, but it suspected that they are reduction products of palmitic and stearic acid.

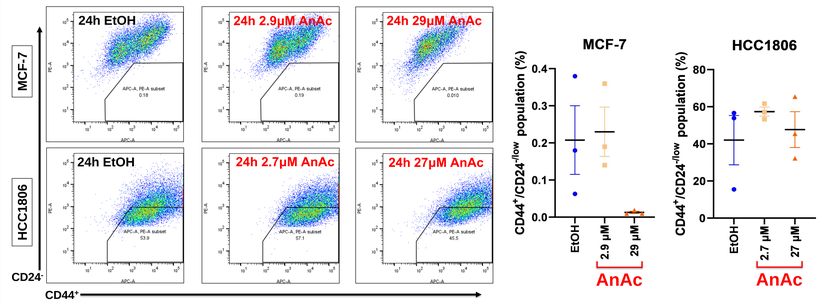


Figure 6: Initial flow cytometry data (n=3) for CSC in MCF-7 and HCC1806 cells reveals a higher % CSC in the HCC1806 TNBC cell line. Images are the median sample values. More replicates are needed to obtain conclusions.

CONCLUSIONS

- Identified the number of metabolite changes in response to AnAc out of the total 193 metabolites:

Cell Line	# of Metabolite Changes
MCF-7	33
BT-20	21
HCC1806	27
MDA-MB-231	28
MDA-MB-468	14

- Identified the top pathways associated with AnAc-induced changes in metabolites **(Table 2)**
- Each TNBC cell line is *unique* in its metabolic response to both AnAc and EtOH control treatments
- AnAc reduced SCD protein expression in MDA-MB-231 and MDA-MB-468 cells and increased SCD protein expression in HCC1806 cells **(Figure 4)**
- Higher SCD increases the conversion of stearic to oleic acid, but the conversion of palmitic to palmitoleic acid was only confirmed in MDA-MB-231 and MDA-MB-468 cells **(Figure 5)**
- High oleic acid in HCC1806 TNBC cells corresponds with the higher % CSC compared to MCF-7 cells.

This work was supported by a grant from the James G. Brown Cancer Center to CMK.
CP was supported by NIH R25 CA134283-09 to David W. Hein.