Targeting Glutamine Metabolism in Breast Cancer with Aminooxyacetate

Preethi Korangath¹, Wei Wen Teo¹, Helen Sadik¹, Liangfeng Han¹, Noriko Mori², Charlotte M Huijts¹, Flonne Wildes², Santosh Bharti², Zhe Zhang¹, Cesar A. Santa-Maria¹, Hualing Tsai¹, Chi V. Dang³, Vered Stearns¹, Zaver M. Bhujwalla², Saraswati Sukumar¹

¹Department of Oncology, ²Department of Radiology, Johns Hopkins University School of Medicine, Baltimore, MD-21231, ³Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA 19104

Running title: Targeting glutamine metabolism in breast cancer

Key words: Aminooxyacetate, glutamine, aspartic acid, breast cancer, c-myc, endoplasmic reticulum stress.

Financial support: This research was supported by Department of Defense Center of Excellence grant W81XWH-04-1-0595 (to S.S); Susan Komen Foundation for the Cure Postdoctoral grant (PDF12231403; to P.K.); the Cindy Rosencrans Fund for Triple Negative Breast Cancer Research (to V.S), and the SKCCC Core grant P30 CA006973 (to S.S).

Correspondence to: Saraswati Sukumar, Ph.D.
Breast Cancer Program
Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins
1650 Orleans Street, CRB-I Rm 143
Baltimore, MD 21231-1000

The authors declare no conflict of interest

Word count: 4997 from Introduction to Fig legends

Abstract: 250 words

No: of figures: 6
Statement of translational relevance: The transaminase inhibitor, aminooxyacetate, targets glutaminolytic pathways and displays potent antitumor effects in preclinical models of c-MYC overexpressing ER-positive and ER-negative breast cancer. Knowledge of the mechanism of action, effectiveness and tolerability of aminooxyacetate make it an excellent candidate for further clinical translation.
Abstract

Purpose: Glutamine addiction in c-MYC-overexpressing breast cancer is targeted by the aminotransferase inhibitor, aminooxyacetate (AOA). However the mechanism of ensuing cell death remains unresolved.

Experimental Design: A correlation between glutamine dependence for growth and c-MYC expression was studied in breast cancer cell lines. The cytotoxic effects of AOA, its correlation with high c-MYC expression, and effects on enzymes in the glutaminolytic pathway were investigated. AOA-induced cell death was assessed by measuring changes in metabolite levels by Magnetic Resonance Spectroscopy (MRS), the effects of amino acid depletion on nucleotide synthesis by cell cycle and BrdU uptake analysis, and activation of the endoplasmic reticulum (ER)-stress mediated pathway. Antitumor effects of AOA with or without common chemotherapies were determined in breast cancer xenografts in immunodeficient mice and in a transgenic MMTV-rTtA-TetO-myc mouse mammary tumor model.

Results: We established a direct correlation between c-MYC overexpression, suppression of glutaminolysis and AOA sensitivity in most breast cancer cells. MRS, cell cycle analysis and BrdU uptake measurements indicated depletion of aspartic acid and alanine leading to cell cycle arrest at S-phase by AOA. Activation of components of the ER stress-mediated pathway, initiated through GRP78, led to apoptotic cell death. AOA inhibited growth of SUM159, SUM149, and MCF-7 xenografts and c-myc overexpressing transgenic mouse mammary tumors. In MDA-MB-231, AOA was effective only in combination with chemotherapy.

Conclusions: AOA mediates its cytotoxic effects largely through the stress response pathway. The preclinical data of AOA's effectiveness provides a strong rationale for further clinical development, particularly for c-MYC overexpressing breast cancers.
Introduction

Metabolic alterations have recently emerged as one of the hallmarks of cancer (1). Cancer cells undergo more rapid replication and therefore have high biosynthetic and bioenergetic demands. To meet this increasing demand, nutrient uptake and metabolic pathways are altered in cancer cells. Even in the presence of adequate oxygen, cancer cells depend on glycolysis rather than oxidative phosphorylation for energy (2). Recent studies in cancer metabolism have shed light on the role of alternative energy sources, especially glutamine and other amino acids, in cell proliferation and maintenance (3-5), including participation of oncogenes and tumor suppressor genes in regulating metabolic pathways in cancer cells (6-8).

Recent findings indicate that increased transcriptional activity of c-MYC is a characteristic feature of triple negative breast cancer (TNBC) (9). High c-MYC alters glutamine catabolism, which significantly enhances glutamine uptake, and shifts glutamine metabolic pathways to maintain redox-balance and fuel energy for cell growth (10), rendering it a novel therapeutic target (11). Aminooxyacetate (AOA) is a general inhibitor of pyridoxal phosphate (PLP)-dependent enzymes including transaminases that are involved in amino acid metabolism and has displayed significant antitumor effects as a single agent in preclinical studies (10, 12, 13). In clinical trials of patients with tinnitus (14, 15) and Huntington’s disease (16), AOA was well tolerated at approximately 1-2mg/kg/day. At these levels, AOA increased levels of circulating and urinary amino acids (15, 16).

We speculated that AOA inhibits cell growth by depleting the amino acid pool via inhibition of transaminases. ER stress is then triggered by amino acid deprivation, leading to the activation of unfolded protein response (UPR) (17). A persistent activation of the ER stress pathway will lead to induction of apoptosis (18).
Here, we report the results of a preclinical study of AOA’s mode of action and antitumor effects in xenograft models of breast cancer in immunodeficient mice, and in an immune-efficient c-myc transgenic mouse mammary tumor model. The data supports a therapeutic role for AOA as a metabolic inhibitor, particularly in c-MYC overexpressing breast cancer.

Materials and Methods

Cell lines and reagents. Breast cancer cell lines used were those frozen within 6 months of purchase from ATCC (authenticated using STR profile analysis) and are as follows: MCF-7 (ER/PR+ve/HER2-negative); SKBR3, HCC1954, HCC202 (ER/PR-negative/HER2+ve); BT474 (ER/PR+/HER2+ve); MDA-MB-231, HCC1806, HCC1143 (from ATCC), SUM149 and SUM159 (S. Ethier, MUSC, SC) (ER/PR/HER2-negative or triple negative). These two cell lines were not authenticated independently. Aminooxyacetate and biochemicals were purchased from Sigma. Normal human mammary epithelial cells were isolated from reduction mammoplasty samples and grown in MCF10A medium (ATCC). Human breast organoids were prepared by enzymatic digestion of reduction mammoplasty tissue, collected under IRB approved protocols. Mouse tumor cell lines, MTC1 and MTC2 were established from primary mammary tumors in doxycycline induced MMTV-rTtA-TetO-myc mice, while MG1 and MG2 were primary mammary gland from FVB/n litter mates.

MTT assay. Cells were plated in 96 well plates at 1500-5000 cells per well in 100 µl media. New medium with varying concentration of AOA was added after 12 h. The assay was performed after 48h (19).

Aspartate transaminase assay. Enzyme activity of aspartate transaminase was measured by a colorimetric assay assessing formation of pyruvate from oxaloacetate, a product of GOT1/2 (also called AST1/2) activity, as described previously (20). In brief, cells grown in 6-well plates were collected after 6, 24 or 48h of AOA treatment and washed with cold PBS, lysed, and supernatant used for analysis.
Western blot analysis. Antibodies used were as follows: anti-c-MYC (Abcam), GRP78, PERK, IRE1a, CHOP, pAMPK, TAMPK, PARP, c-PARP, c-Cas3 (Cell Signaling), cyclin D1, ATF3, (Invitrogen), β-actin (Sigma). Quantitation was performed using Image J software.

Magnetic resonance spectroscopy (MRS). SUM159 cells were treated with AOA for 24h. Adherent cells were collected by trypsinization and live cells were counted. Water-soluble as well as lipid extracts were obtained using the dual-phase extraction method (21).

Cell cycle analysis and BrdU incorporation assay. SUM149 or SUM159 cells were trypsinized after treatment and fixed in 80% ethanol overnight at −20°C. Subsequently, cells were resuspended in a PBS solution containing 20 μg/mL propidium iodide (PI) and 10 μg/mL RNase A and incubated 30 minutes at 37°C(22). For BrdU incorporation, cells were treated with BrdU (Invitrogen) for 60 min at 37°C and washed twice with PBS(23).

Animal experiments. All animal studies were approved by the Institutional Animal Care and Use Committee at Johns Hopkins University. Two million SUM149, SUM159, HCC1954, MDA-MB-231 or 5 million MCF-7 cells resuspended in 50 µl PBS and matrigel (BD Bioscience) (1:1) were injected subcutaneously to athymic Balb/c mice (NCI, Frederick). Tumors were measured twice a week.

Transgenic mouse model: Doxycycline-inducible, mammary-specific myc-overexpressing mice (MMTV-rTtA-TetO-myc) were generated as previously described (24).

Statistical analysis. Quantitative data were analyzed using GraphPad Prism (v5.0, GraphPad Software, San Diego, CA) and SAS software (v9.2, SAS Institute, Cary, NC), and expressed as mean ± standard deviation (SD) or percent when appropriate.

Detailed description for each section is provided in Supplementary Methods.
Results

Glutamine dependence of breast cancer cells for growth. The dependence of breast cancer cells to glutamine has not been extensively cataloged. Cells were cultured either in growth medium supplemented with 10% FBS that contained glutamine or was glutamine-free (designated reduced glutamine medium). The growth of breast cancer cell lines MDA-MB-231, SUM149, HCC1806, SUM159, and MCF-7 was dependent on glutamine supplementation. In contrast, BT474, HCC143, HCC1954, HCC202 and SKBR3 were significantly less dependent on glutamine for growth, as assessed by trypan blue staining (Fig.1A), and also confirmed by colony formation assays (Supplementary Fig.S1A). The number of apoptotic cells increased significantly in glutamine dependent MDA-MB-231 and SUM149 cell lines grown with reduced glutamine for 6 days, while no such change was observed in glutamine independent BT474 or HCC202 cell lines (Supplementary Fig.S1B).

Differential expression of genes in the glutaminolytic pathway, and sensitivity to AOA.
Glutamine provides carbon moieties to the TCA cycle through its entry into mitochondria as alpha-ketoglutarate, either through the activity of glutamate dehydrogenase (GDH) or glutamic oxaloacetate transaminases (GOTs). Nitrogen from glutamine is essential for biosynthesis of nucleotides and of amino acids glutamate and aspartate, which are the precursors for the synthesis of other nonessential amino acids (Schema in Fig.1B) (25).

The variable dependence of cancer cells on glutamine could be the consequence of altered expression of genes involved in the glutaminolytic pathway. By RT-qPCR, levels of GOT1, GOT2, GPT2 and GLS2 (Fig.1C), but not GLS1 and GDH (Supplementary Fig.S1C) were significantly higher in breast cancer cell lines compared to normal epithelial organoids and cultured HMECs. Interestingly, GPT2 expression was significantly higher in glutamine dependent cell lines compared to glutamine independent cell lines (Fig.1C). Upon glutamine withdrawal for 24h, the expression of GOT1 increased significantly in MDA-MB-231 and
SUM149 cells (Fig.1D, Supplementary Fig.S1D), but remained unchanged in glutamine independent BT474 and HCC202, suggesting GOT1 is one of the key enzymes in glutaminolysis. GPT2 catalyzes a reversible transamination reaction to yield alpha-ketoglutarate and alanine from pyruvate and glutamate. GPT2 mRNA was higher in all three glutamine-dependent cell lines compared to -independent cell lines (Fig.1D). These results suggested that elevated GPT2 is also a key enzyme that helps fuel cells to enable survival in a glutamine-poor environment. No specific inhibitors for GOT or GPT2 are currently available. Hence we tested the effects of pan transaminase inhibition using AOA on survival of 10 ER+ and ER- breast cancer cell lines grown in complete medium. Glutamine-dependent cell lines showed greater inhibition of cell growth by AOA compared to cells that were less glutamine-dependent (Fig.2A and Supplementary Fig.S2A). To identify which enzyme activity is targeted by AOA, we used SUM149 cell lines depleted of each enzyme (using siRNA) and tested their sensitivity towards AOA. As shown in Fig.2B, depletion of GOT1, GOT2 or GPT2 partially reversed AOA-mediated cytotoxic effects. When cells were depleted of GOT1 plus GOT2, or GOT1/2 plus GPT2, cells showed lowered sensitivity to AOA. Interestingly, when cells were depleted of GPT2, both GOT1 and GOT2 levels showed up to 1.6 fold increase (Fig.2C). These results led to the conclusion that GOT1/2 and GPT2 are partially responsible for AOA-mediated effects, and also suggested compensatory effects following GPT2 depletion, whereby knocking down this enzyme caused the cells to increase the levels of the others. Moreover, cell proliferation was also affected by the knockdown of these genes either alone or in combination in SUM149 cells, which emphasizes the importance of these genes in cell survival (Supplementary Fig.S2B). The enzymatic activity of GOT1/2 tested in three cell lines was also significantly inhibited by AOA in a time-dependent manner (Fig.2D).

AOA effect on breast cancer cells is c-MYC-dependent. Among the earliest molecular aberrations identified in breast cancer, c-MYC amplification and overexpression was reported to
occur in 30-50% of breast cancers (26). The global effects of c-MYC on metabolism, especially on glutamine metabolism, have been previously studied (27, 28). c-MYC transcriptionally activates the glutamine transporters and glutaminases (29). The role of c-MYC in glutamine addiction has been well studied in glioblastoma (30) and osteosarcoma (10), but its effect on breast cancer metabolism is not well established. Western blot analysis in a panel of cell lines showed that c-MYC levels are higher than normal in 50% of the tumor cells, and c-MYC levels correlated with glutamine dependence and AOA sensitivity, with a Spearman correlation of r=0.664; p=0.03 (Fig.3A). To test the importance of high c-MYC in AOA action, we generated TNBC cells depleted of c-MYC. Transient knockdown of c-MYC in SUM149 and SUM159 (Fig.3B), and stable depletion in SUM159 cells (Fig.3B) rendered the cells significantly less sensitive (p<0.05) to AOA-mediated cell death. Compared to control cells, SUM159-sh-cMYC cells were less glutamine-dependent for growth (Fig.3B) and showed a significantly lower expression of glutaminolytic genes, GOT1 and GPT2, but not GDH or GOT2 (Supplementary Fig.S3A). Conversely, overexpression of c-MYC in HCC1954 (low c-MYC) increased AOA sensitivity (p<0.05; Fig.3C). Collectively, these results demonstrate that AOA sensitivity was significantly greater in cells with high c-MYC expression. This finding was validated using two mouse mammary tumor cell lines, MTC1 and MTC2, established from doxycycline-induced MMTV-rTtA-TetO-myc-driven mammary tumors in FVB/N mice. Both cell lines express high c-myc (maintained in doxycycline-containing medium), were found to be glutamine dependent (p<0.001; Supplementary Fig.S3B, C), and were sensitive to growth inhibition by AOA (p<0.05; Fig.3D). Taking advantage of the inducible c-myc present in these cells, we tested the importance of c-myc expression in their response to AOA. A progressive decrease in expression of c-myc in MTC2 cells was achieved by withdrawal of doxycycline from the culture medium. As the c-myc levels decreased over time, the MTC2 cells showed significant loss of sensitivity towards AOA by day 12 (Supplementary Fig.S3D), supporting a significant role of high c-myc expression in the response of the breast cancer tumor cells to AOA.
Magnetic Resonance Spectroscopic (MRS) analysis of metabolic changes in AOA-treated cells. Quantitative measures of metabolite alterations in SUM159 cells treated with AOA by MRS (31) (metabolite spectra, Supplementary Fig.S4A) showed a significant reduction in aspartate and alanine, and no significant change in glutamate content (Fig.4A), suggesting AOA-mediated inhibition of aspartate and alanine transaminases. Following AOA treatment, we also observed a reduction in two metabolites associated with neoplastic transformation, total choline (phosphocholine(PC) + glycerophosphocholine(GPC) + freecholine) and PC(32) (Supplementary Fig.S4B). Ruling out an effect of AOA on the glycolytic pathway, lactate production and glucose consumption measured in the conditioned media collected from AOA treated-SUM159 cells showed no significant changes (Supplementary Fig.S4C). Thus, MRS analysis further confirmed AOA action in depleting alanine and aspartate and detected a reduction in the established markers of neoplasia, total choline and PC.

MRS data suggested that AOA causes depletion of both aspartate and alanine, which are amino acids critical for the growth of many breast cancer cells. If so, exogenous addition of these amino acids should rescue cells from AOA-induced cell death. Cultured MDA-MB-231, SUM149 and SUM159 cells were treated with varying doses of AOA in the presence or absence of alanine or aspartate. Under our experimental conditions, alanine did not rescue cells from AOA-induced toxicity (Supplementary Fig.S4D,E), while aspartate rendered cells less sensitive to AOA (Fig.4B). These data suggested that cell death caused by AOA occurs mainly through aspartate depletion.

AOA caused cell cycle arrest at S phase, reversed by aspartate. Aspartate is an amino group donor in nucleotide biosynthesis. Optimal nucleotide levels are necessary for entry into the cell cycle (33). Depletion of aspartate by AOA could, therefore, compromise nucleotide biosynthesis causing cell cycle arrest at S-phase. Approximately 13-15% of exponentially growing SUM159 (Fig.4C) and SUM149 cells (Supplementary Fig.S5A) are in S-phase. With
AOA treatment, an increase in S-phase fraction (32% and 38% respectively) and a concomitant decrease of cells in G1 phase was observed. Exogenous aspartate reversed S-phase arrest induced by AOA. The sub-G1 fraction representing apoptotic cells was also significantly increased in AOA treated cells (Fig.4C and Supplementary Fig.S5A). To evaluate the stability of S-phase arrest, SUM159 cells were pulsed with BrdU and treated with either fresh medium or with 2mM AOA. Control cells gradually lost incorporated BrdU with each doubling. In AOA treated cells, however, the number of BrdU-positive cells remained unchanged for 72h, indicative of a persistent cell cycle arrest (Fig.4D). Similar results were obtained with SUM149 cells (Supplementary Fig.S5B). Furthermore, AOA-induced cell cycle arrest was irreversible, since continued treatment of SUM149 and SUM159 cells for 10 days induced cell death (Supplementary Fig.S5C,D). We also evaluated whether exogenous aspartate could substitute glutamine, and reverse cell death. Glutamine dependent MDA-MB-231, SUM149, SUM159 and HCC1806 cell lines were cultured in normal medium, medium with reduced glutamine, or medium with reduced glutamine supplemented with exogenous aspartate. Exogenous aspartate was able to partially rescue cells from glutamine deprivation-mediated cell death in these cell lines (Supplementary Fig.S5E).

**Mechanism of AOA-mediated apoptotic cell death.** MRS analysis of metabolites showed that AOA action occurs, in large part, by blocking amino acid metabolism. This, in turn, is known to induce AMPK activation and ER stress in cells. Persistent ER stress leads to cell death through apoptosis (18). To examine these pathways, we treated SUM149 and SUM159 cells with AOA for 24h and evaluated mRNA expression and protein analysis by RT-qPCR and immunoblotting analysis. AOA treatment significantly increased the mRNA levels of ER stress markers such as ATF3, CHOP, but not PERK or ATF6 (Fig.5A). Next, we analyzed whether these alterations are specific to AOA-sensitive cell lines. Western blots analysis of the ER stress markers IRE-1a, GRP78, CHOP and the apoptosis indicator, cleaved caspase 3, following AOA
treatment (2mM, 72h) showed significant increases in levels of stress markers in SUM159 cells compared to control BT474 or HCC202 cell lines (Fig.5B). GRP78, the ER stress modulator and chaperone for three key sensors, ATF6, PERK and IRE-1a, showed a 30% decrease in SUM159 cells. A three-fold increase of IRE-1a (transducer of the ER stress signaling following its dissociation from GRP78), and 12-fold increase in CHOP (the apoptotic inducer in the ER stress pathway) was observed following AOA treatment. A 15-fold increase in cleaved caspase 3 was detected in SUM159 cells. This data supported specificity of AOA-mediated effects in glutamine-dependent, but not in glutamine independent cell lines. In addition, Cyclin D1 levels decreased within 24h, and AMPK was phosphorylated. Levels of ATF3, acting upstream of CHOP, increased in SUM159 and SUM149 cells. PARP cleavage, another indicator of cell death, was also detected (Supplementary Fig.6A,B). Addition of aspartate in SUM159 cells partially reversed AOA-induced increase in IRE-1a and CHOP, and decrease in levels of GRP78 and cleaved caspase 3 (Fig.5C).

We also examined the ER stress markers in MDA-MB-231 cells following treatment with AOA combined with the common chemotherapeutic, carboplatin. Carboplatin induced no significant effect on the stress markers, but compared to AOA alone, the combination showed an additive effect on increases in both mRNA and protein levels. The other markers also showed a similar pattern (Supplementary Fig.6C,D). Overall, these data provided strong evidence for induction by AOA of a robust ER stress response through GRP78, IRE-1a or CHOP, which likely accounts for cell death observed following treatment. A schema outlining the proposed mechanism of action of AOA in c-MYC overexpressing cancer cells is shown in Fig.5D.

**Antitumor effects of AOA in animal models.** Cell culture analysis showed that AOA has growth inhibitory effects, in particular on cells that express high levels of c-MYC protein. We tested the antitumor effects of AOA on xenografts of three high c-MYC expressing SUM149, SUM159 and MCF-7 breast cancer cell lines, and a low c-MYC expressing cell line, HCC1954
as a negative control. Treatment with AOA (5mg/kg) resulted in a significant reduction in tumor growth in SUM149 (p<0.01), SUM159 (p<0.001) (Fig.6A and Supplementary Fig.S7A,B) and MCF-7 (p=0.001) (Supplementary Fig.S7C) xenografts compared to vehicle control but not in low MYC-expressing, negative control HCC1954 tumors (Fig.6A). Immunohistochemical analysis of AOA-treated SUM159 and SUM149 tumors showed higher levels of cleaved caspase 3 (Supplementary Fig.S7D). Protein levels of ER stress markers PERK, IRE1 and CHOP were higher in both SUM159 and SUM149 tumors treated with AOA but not in HCC1954 xenografts (Fig.6B and supplementary Fig.S7E,F,G). Next, we tested the effect of AOA in combination with carboplatin and paclitaxel, drugs commonly used in the clinic to treat TNBC. No loss of body weight occurred in the immunodeficient recipient mice with AOA treatment (Supplementary Fig.S8A). The combination of AOA with paclitaxel significantly inhibited SUM149 tumor growth compared to paclitaxel alone (Supplementary Fig.S8B) but carboplatin had no additive effect (Supplementary Fig.S8C). Chemotherapy did not enhance the antitumor effects of AOA in SUM159 tumors (Supplementary Fig.S8D,E).

An exception to these observations was noted in MDA-MB-231 cells. Here, despite expression of c-MYC at moderate levels, in culture, cells were very sensitive to AOA. In addition, activation of ER stress and apoptotic pathways occurred in MDA-MB-231 cells treated with AOA alone, with a more pronounced effect when AOA was combined with carboplatin (Supplementary Fig.S6C,D). Treated with AOA as a single agent, MDA-MB-231 xenografts did not show any growth inhibition (Fig.6C). Here, combinations of AOA with doxorubicin, carboplatin, or paclitaxel resulted in significant (p<0.01) reduction in size of tumors. Western blot analysis of a representative set of tumors showed that treatment with carboplatin or doxorubicin alone resulted in a nearly 2-fold increase in the level of c-MYC protein compared to either vehicle control or AOA treatment (Fig.S8F). These data raised the possibility that susceptibility to AOA correlates not only with constitutive, but also with chemotherapy-induced overexpression of c-
MYC protein. Also, effectiveness of treatment with AOA as a single agent or in combination with chemotherapy may be predicted by c-MYC levels in the pretreatment tumor biopsy. In addition, post-treatment upregulation of c-MYC could contribute to greater sensitivity of AOA.

To further substantiate our findings, we used the doxycycline-inducible MMTV-driven myc overexpressing transgenic mouse model. Upon doxycycline induction, the bitransgenic mice develop mammary tumors with a latency of around 22 weeks (24). When tumors reached a size of 150-200mm³, mice were treated with AOA at 0.5mg/kg body weight/3 days a week for 4 weeks (n= 5-10/group). There was a significant reduction in tumor growth in mice treated with AOA (Fig.6D). In contrast to Balb/c nu/nu mice, doses of AOA above 0.5mg/kg (doses tested, 0.5, 1, 2.5 and 5mg/kg) caused loss of body weight and death in this strain of mouse.

Discussion

The role of glutamine metabolism on proliferation has not yet been studied extensively in breast cancer, particularly in TNBC. In this study, we have provided evidence that breast cancer cell lines that express high levels of c-MYC are dependent on glutamine for their survival and growth. Suppression of glutaminolysis in these cell lines using a transaminase inhibitor, AOA, resulted in cell death, largely through activation of the ER-stress pathway. These findings led us to further develop AOA in breast cancer as a therapeutic target.

Inhibition of glutamine metabolism is an active area of research. As a result several agents targeting glutamine metabolism are under development (34). In 2008, researchers reported that treatment of a single cell line, MDA-MB-231 with AOA resulted in a decrease in alanine and flux of ¹³C-glucose-derived carbons into glutamate and uridine, and a reduction in oxygen consumption rate, cellular ATP level and NAD+/NADH ratio (13). In this study we have reported an in depth characterization of AOA-mediated cell death pathways in breast cancer cells. The expression level and activity of many enzymes essential for glutamine metabolism including GOT1, GOT2, GPT2 and GLS2 was highly elevated in breast cancer cell lines.
compared to normal breast cells. This reliance on alternative sources of energy, in all likelihood, forms the basis for the addiction of the breast cancer cells to glutamine. A second factor contributing to glutamine addiction could be high levels of c-MYC expression in cells (30). If so, it stands to reason that c-MYC overexpressing cells will be particularly sensitive to transaminase inhibitors such as AOA. In fact, we found a significant correlation between c-MYC level and drug sensitivity (Fig.3A-C). Our data are consistent with published findings where ATF4 and its downstream regulators were found to be critical mediators of apoptosis consequent to glutamine withdrawal in c-MYC overexpressing neuroblastoma (12). In TNBC cells, AOA treatment lowered levels of the chaperone protein, GRP78, and activated many ER stress pathway genes including ATF3, downstream of ATF4. Stresses in the tumor microenvironment such as low oxygen, low glucose and decreased amino acid availability activate UPR, a cellular homeostatic program triggered by an excess of misfolded or unfolded proteins in the ER lumen (18). During prolonged stress UPR initiates a program leading to apoptosis. The three proximal effectors of the UPR are pancreatic ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring transmembrane kinase/endoribonuclease 1 (IRE1). Autophosphorylation of PERK permits the translation of specific cap-independent ER stress response genes, such as ATF4. The proapoptotic protein CHOP (CCAAT/-enhancer-binding protein homologous protein), is upregulated downstream of ATF4, causes downregulation of the anti-apoptotic mitochondrial protein Bcl-2, promoting apoptosis. Secondly, ATF6 is activated by proteolytic cleavage following translocation to the Golgi. (35). IRE1α activates a JNK signaling pathway, at which point human procaspase 4 is believed to cause apoptosis by activating downstream caspases (18). The mammalian adenosine monophosphate–activated protein kinase (AMPK) is a serine-threonine kinase protein complex that is a central regulator of cellular energy homeostasis; the mechanisms by which AMPK mediates cellular responses to metabolic stress remain unclear (36). We have shown here that ER stress may constitute a major pathway in cell growth inhibition caused by AOA treatment.
We also propose a novel mechanism of cell growth inhibition by AOA. It is well-known that glutamine and aspartate provide amine group that are critical for nucleoside synthesis. The reduction in the nucleoside pool in the cell may cause cell cycle arrest in S phase as shown by our cell cycle analysis and BrdU incorporation assay (37). In our study, aspartate alone effectively decreased AOA sensitivity to breast cancer cells and reversed S phase arrest, which could be attributed possibly to the rescue of nucleotide synthesis by aspartate. Another interesting finding was the effect of AOA on choline metabolism as observed in the MRS studies. Elevated levels of PC and total choline have been reported in malignant breast tumors (32, 38, 39). The role of AOA in decreasing total choline and phosphocholine remains to be investigated.

Although MDA-MB-231 cells were sensitive to AOA in vitro, contrary to the published report (13) which observed the AOA treated mice (10mg/kg body weight) for two weeks, we did not observe tumor growth inhibition at 5mg/kg body weight of AOA. In our hands, larger doses were associated with loss of weight and signs of toxicity after a week, especially when combined with chemotherapeutic agents. Whether MDA-MB-231 cells overcome AOA-induced ER stress-mediated cell death in vivo through cross talk with the microenvironment needs further investigation.

AOA had a potent growth inhibitory effect on MMTV-rTtA-TetO- myc transgenic mouse mammary tumors. By taking advantage of the doxycycline inducibility of the gene in this system, we demonstrated that myc depletion reverses AOA susceptibility of the tumor cells. These findings, in addition, provide a strong rationale for exploring the utility of this small molecule in c-MYC overexpressing breast tumors. AOA may be an appealing therapeutic agent based on its high tolerability and strong antitumor effects, providing a strong rationale for further drug development.
In summary, we have shown that breast cancer cells, particularly TNBCs, are dependent on glutamine for growth and this dependence can be effectively targeted by AOA. The ER stress pathway induced by AOA leads to cell growth inhibition and apoptosis. AOA, while cytotoxic to tumor cells, has shown acceptable toxicity profiles in small clinical trials, which may allow for rapid drug development. Furthermore, since pre-clinical data suggest a role for glutamine metabolism in multiple other cancer types, specifically sarcomas, brain tumors, and other c-MYC driven cancers, AOA may have a wider therapeutic application.

Acknowledgements

We thank Dr. Lewis Chodosh, University of Pennsylvania for generously providing MMTV-rTtA-tetO-myc transgenic mice and Dr. A. Goga for providing the c-MYC overexpression vector. We thank Dileep Unnikrishnan and Sidra Hafeez for laboratory assistance.

References


**Figure Legends**

**Figure 1. Glutamine dependency of breast cancer cell lines**

(A) Breast cell lines were grown in media with complete or reduced glutamine (Gln and Gln-R).

The average number of live cells/well (3 wells/time point; 2 replicates), on indicated days is shown.

(B) Schematic representation of transaminases and other genes involved in glutaminolysis.

(C) Differential expression of GOT1, GOT2, GLS2 and GPT2 in breast cancer cell lines compared to normal mammary organoids and HMECs (duplicates in each assay; 3 replicates). Mann Whitney test, *p<0.05.

(D) RT-qPCR of genes in the glutaminolytic pathway following growth in medium with complete (Gln +) or reduced glutamine (Gln -) for 24h (duplicates in each assay; 3 replicates). Mann Whitney test, *p<0.05.
Figure 2. Sensitivity of breast cancer cells to AOA and its reversal in cells depleted of GOT1/2 and GPT2

(A) MTT assay of breast cancer cell lines and normal human mammary epithelial cells (HMEC) after treatment with 2mM AOA for 48h.

(B) Reversal of cytotoxic effects of AOA in SUM149 cells depleted of GOT1, GOT2 and GPT2.

(C) RT-qPCR of GOT, GOT2 and GPT2 mRNA in SUM149 cells depleted of the indicated genes.

(D) Enzymatic activity of GOT1 and GOT2 after AOA treatment (2mM) shows significant inhibition following 6 h of exposure.

Each experiment was repeated 3 times. All statistical analysis by Student t-test; 2-tailed; *p<0.05

Figure 3. AOA sensitivity is c-MYC dependent

(A) Western blot analysis of c-MYC protein in breast cancer cells; densitometry evaluation normalized with loading control β-actin; and correlation between c-MYC protein level and sensitivity to AOA. The percent cells surviving after 48 hr of treatment with 2 mM AOA (from Fig. 2A) was plotted against quantity of c-MYC protein in the same cell line. Spearman correlation r=0.664; *p<0.05.

(B) siRNA mediated depletion of c-MYC in SUM149 and SUM159 cells. MTT assay of cells treated with AOA for 48h. Sensitivity of SUM159 cells stably expressing c-MYC shRNA compared to scramble (Scr) shRNA expressing cells (left panel) to AOA; colony formation assay showing that MYC-depleted SUM159 cells are not dependent on glutamine for growth (right panel).
(C) MTT assay to test the effect of AOA in HCC1954 breast cancer cells overexpressing exogenous c-MYC.

(D) MTT assay to test the effect of AOA in two mouse mammary tumor cell lines. Western blot shows c-myc overexpression in two tumor cell lines, MTC1 and MTC2, compared to uninduced FVB/N mouse mammary glands, MG1 and MG2.

Values are represented as mean±SD of 3 independent experiments, with 4 wells tested for each condition for panels 3B-D. All the data was analyzed by Student t-test, 2-tailed *p<0.05.

**Figure 4.** (A) MRS analysis of SUM159 cells treated with AOA showing significant decrease in alanine and aspartate, but not glutamate (*p<0.05).

(B) MTT assay showing percent viable cells in culture in the presence of 10mM aspartate with varying amounts of AOA for 48h (*p<0.01).

(C) AOA induces S phase arrest, reversed by adding aspartate. Flow cytometry analysis of cell cycle in SUM159 cells exposed to vehicle, AOA, aspartate or AOA+aspartate for 72h using propidium iodide showing increase in sub-G1 content and S-phase arrest in AOA-treated cells; this effect was reversed by co-treatment with aspartate.

(D) Stability of S-phase arrest by BrdU incorporation and retention. Cells were stained for BrdU and PI, and analyzed by flow cytometry showed significant increase of BrdU positive cells after 48 and 72h of AOA (2mM) treatment.

Values are represented as mean±SD of 3 independent experiments for panel 4A-D. All the data was analyzed by Student t-test, 2-tailed.

**Figure 5. Molecular mechanism of AOA-induced growth inhibition and death**
(A) RT-qPCR analysis of SUM159 and SUM149 cells showing induction of ER stress markers after 24h exposure to 2mM AOA (duplicates in each assay; 3 replicate assays). Student t-test, 2-tailed p=<0.05

(B) Western blot analysis for stress markers and apoptotic marker caspase3 comparing AOA sensitive SUM159 with AOA-insensitive BT474 and HCC202 cell lines after 72h exposure to 2mM AOA.

(C) Induction of ER stress markers and caspase3 is reversed by addition of aspartate to SUM159 and SUM149 cells following 48h or 72h of treatment with AOA. Bar graph shows quantitation.

(D) Schema of proposed mechanism of action of AOA in breast cancer cells.

**Figure 6. In vivo growth inhibitory effect of AOA**

(A) SUM149, SUM159 or HCC1954 cells were injected s.c into athymic Balb/c mice. When tumors reached a size of 100mm³, mice were randomized to control (PBS) or AOA (5mg/kg i.p, daily) treatment (n=6-8 mice per group).

(B) Western blot analysis of stress pathway markers in xenografts of SUM159, SUM149 and HCC1954. Tumors were collected at the end of experiment.

(C) In MDA-MB-231 xenografts, a combination of AOA with doxorubicin, carboplatin or paclitaxel showed growth inhibitory effects.

Data are plotted as the mean±SEM and compared via mixed-effects models with Tukey’s procedure for multiple testing corrections.

(D) (Left panel) Tumor bearing MMTV-rTtA-TetO-myc mice were treated with PBS (n=10) or AOA 0.5mg/kg (i.p 3 days/week) (n=5). Treatment was initiated when the tumors reached 150-
200 mm$^3$ and was continued for 4 weeks. (Right panel) Body weight of animals receiving AOA showed no significant decrease compared vehicle treated animals. ANOVA; $p=0.001$. 
Figure 1

A. Number of cells \(\times 10^3\) over days for different cell lines:

- **HCC1143**
- **SKBR3**
- **HCC1806**
- **SUM159**
- **MCF-7**
- **BT474**
- **HCC202**

B. Metabolic pathways in cytoplasm and mitochondria:

- **GLS1**
- **GDH**
- **GLS2**
- **AST1/GOT1**
- **AST2/GOT2**
- **TCA cycle**
- **Glutamine**
- **Glutamate**
- **aKG**
- **malate**
- **OAA**
- **Citrate**
- **Ac-CoA**
- **Lipids**
- **Pyruvate**
- **Alanine**

C. Relative fold change for enzyme activities:

- **GOT1**
- **GOT2**
- **GPT2**
- **GLS2**

D. RTqPCR results:

- **GOT1**
- **GPT2**
- **GLS2**
Figure 2

A.

Gln-independent

Gln-dependent

Percent Survival

HMEC  HCC-202  Br474  SkBR3  HCC1954  HCC 1143  MCF7  HCC1966  SUM159  SUM149  MDA MB-231

p=0.002

B.

Percent Survival

AOA 2 mM

SUM149

p<0.0001

p<0.0001

p<0.0001

p<0.0001

p<0.0001

C.

Fold change

GOT1

GOT2

D.

GOT1/2 activity assay

MDA-MB-231  SUM149  SUM159

Percent Inhibition of activity

Hours

0  6  24  48

*p  **  ***

*  **  ***
Figure 4

A. Relative amount of Alanine, Aspartate, and Glutamate in Control and AOA 2mM treatments.

B. Percent Survival of SUM 159, SUM 149, and MDA-MB-231 cell lines with varying AOA concentrations.

C. Flow cytometry histograms showing FL2-A counts in control, AOA, Asp, and AOA + Asp treatments for SUM159.

D. Percent cells in Sub-G1, G1, S, and G2/M phases, and percent BrdU positive cells for SUM159 with different treatments and time points.
Figure 5

A.

B.

C.

D.

C-myc → ↑ Glutamine metabolism

GOT1 and 2, GPT2

No AOA

AOA

Cell survival

GOT1/2, GPT2

Aspartate, Alanine

ER stress

Apoptosis
Figure 6

A.

SUM149

Tumor volume mm³

Control
AOA (5mg/kg)

p<0.01

HCC1954

Tumor volume mm³

Control
AOA (5mg/kg)

SUM159

Tumor volume mm³

Control
AOA (5mg/kg)

p<0.001

B.

Xenografts: SUM149

PERK
IRE-1a
CHOP
β-actin

Ctrl
AOA

Tumor # 1 2 3 4 5 1 2 3 4 5 6

SUM159

PERK
IRE-1a
CHOP
β-actin

Ctrl
AOA

HCC1954

PERK
IRE-1a
CHOP
β-actin

Ctrl
AOA

C.

MDA-MB-231 xenograft

Control
AOA (5mg/kg)
Carbo (50mg/kg)
AOA + Carbo

p<0.01

AOA + Dox

p<0.01

AOA + Pacli

p<0.01

D.

MMTV-rTtA-TetO-myc mouse mammary tumor model

Control
AOA 0.5 mg/kg

p<0.0001

Control
AOA 0.5mg/kg

Body weight in gms

Weeks

Initial 1 2 3 4

Control
AOA 0.5 mg/kg

p<0.0001

Control
AOA 0.5mg/kg

Initial 1 2 3 4

Weeks
# Clinical Cancer Research

## Targeting Glutamine Metabolism in Breast Cancer with Aminooxyacetate

Preethi Korangath, Wei Wen Teo, Helen Sadik, et al.

_Clin Cancer Res_ Published OnlineFirst March 26, 2015.

<table>
<thead>
<tr>
<th>Updated version</th>
<th>Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-14-1200</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supplementary Material</td>
<td>Access the most recent supplemental material at: <a href="http://clincancerres.aacrjournals.org/content/suppl/2015/03/27/1078-0432.CCR-14-1200.DC1">http://clincancerres.aacrjournals.org/content/suppl/2015/03/27/1078-0432.CCR-14-1200.DC1</a></td>
</tr>
<tr>
<td>Author Manuscript</td>
<td>Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.</td>
</tr>
</tbody>
</table>

---

**E-mail alerts**  
Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**  
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**  
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.