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, and Saraswati Sukumar1

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 bromodeoxyuridine (BrdUrd) 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 BrdUrd 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 provide a strong rationale for further clinical development, particularly for c-MYC–overexpressing breast cancers. Clin Cancer Res; 21(14); 3263–73. ©2015 AACR.

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 energetic demands. To meet this increasing demand, nutrient metabolic pathways are altered in cancer cells. Even in the presence of adequate oxygen, cancer cells depend on glycolysis rather than oxidative phosphorylation for energetic demands. To meet this increasing demand, nutrient uptake and metabolic pathways are altered in cancer cells. 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; ref. 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–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 to 2 mg/kg/d. 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. Endoplasmic reticulum (ER) stress is then triggered by amino acid deprivation, leading to the activation of unfolded protein response (UPR; ref. 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...
support 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 the ATCC (authenticated using STR profile analysis) and are as follows: MCF-7 (ER/PR+/-HER2-negative); SKBR3, HCC1954, HCC202 (ER/PR-negative/HER2+/-); BT474 (ER/PR+/HER2+); 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. AOA and biochemicals were purchased from Sigma. Normal human mammary epithelial cells (HMEC) 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-rTetA-TetO-myc mice, whereas MG1 and MG2 were primary mammary glands from FVB/n litter mates.

MTT assay

Cells were plated in 96-well plates at 1,500 to 5,000 cells per well in 100 µL media. New medium with varying concentration of AOA was added after 12 hours. The assay was performed after 48 hours (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 48 hours of AOA treatment and washed twice with PBS (23).

Western blot analysis

Antibodies used were as follows: anti-c-MYC (Abcam), GRP78, PERK, IRE1a, CHOP, PAMPK, TAMPK, PARP, c-PARP, c-Cax3 (Cell Signaling Technology), cyclin D1, ATF3 (Invitrogen), β-actin (Sigma). Quantitation was performed using ImageJ software.

Magnetic resonance spectroscopy

SUM159 cells were treated with AOA for 24 hours. 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 BrdUrd 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 bromodeoxyuridine (BrdUrd) incorporation, cells were treated with BrdUrd (Invitrogen) for 60 minutes 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 Matriigel (BD Biosciences; 1:1) were injected s.c. to athymic Balb/c mice (NCI, Frederick). Tumors were measured twice a week.

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

Statistical analysis

Quantitative data were analyzed using GraphPad Prism (v5.0, GraphPad Software) and SAS software (v9.2, SAS Institute), and expressed as mean ± SD or the percentage of, when appropriate. Detailed descriptions for each section is provided in Supplementary Methods.

Results

Glutamine dependence of breast cancer cells for growth

The dependence of breast cancer cells on 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, whereas 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. 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; ref. 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 with normal epithelial organoids and cultured HMECs. Interestingly, GPT2 expression was significantly higher in glutamine-dependent cell lines compared with glutamine-independent cell lines (Fig. 1C). Upon glutamine withdrawal for 24 hours, the expression of GOT1 increased significantly in MDA-MB-231 and SUM149 cells (Fig. 1D and Supplementary Fig. S1D), but remained unchanged in glutamine-independent BT474 and HCC202, suggesting that 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 with 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/C0 breast cancer cell lines grown in complete medium. Glutamine-dependent cell lines showed greater inhibition of cell growth by AOA compared with 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 toward

---

**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 per well (3-wells/time point; two replicates), on indicated days is shown. B, schematic representation of transaminases and other genes involved in glutaminolysis. C, differential mRNA expression of GOT1, GOT2, GLS2, and GPT2 in breast cancer cell lines compared with normal mammary organoids and HMECs (duplicates in each assay; three replicates). The 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 24 hours (duplicates in each assay; three replicates); the Mann–Whitney test; *; P < 0.05.
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% to 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 with 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 and S3C), 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 toward 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 analysis of metabolic changes in AOA-treated cells

Quantitative measures of metabolic alterations in SUM159 cells treated with AOA by magnetic resonance spectroscopy (MRS; metabolite spectra, Supplementary Fig. S4A; ref. 31) 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 + glycerophosphocholine + freecholine), and phosphocholine (Supplementary Fig. S4B; ref. 32). 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 phosphocholine.

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 and S4E), whereas aspartate rendered cells less sensitive to AOA (Fig. 4B). These data suggested that cell death caused by AOA occurs mainly through aspartate depletion.

AOA causes 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% to 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
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 (activated protein kinase) 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 24 hours and evaluated mRNA expression and protein levels by RT-qPCR and immunoblotting analysis. AOA treatment significantly increased the mRNA levels of ER stress markers such as ATF3, CHOP, but not PERK (pancreatic ER kinase) or ATF6 (Fig. 5A). Next, we analyzed 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).

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 (5 mg/kg) resulted in a significant reduction in tumor growth in SUM149 (P < 0.001), SUM159 (P < 0.001; Fig. 6A and Supplementary Fig. S7A and S7B), and MCF-7 (P = 0.001; Supplementary Fig. S7C) xenografts compared with vehicle control but not in low MYC-expressing, negative control HCC1954 tumors (Fig. 6A). Immunohistochemical analysis of AOA-treated SUM149 and SUM159 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–S7G). 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 with 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 and S8E).

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 and S6D). 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 with either vehicle control or AOA treatment (Supplementary 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, posttreatment 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 to 200 mm³, mice were treated 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 BrdUrd and treated with either fresh medium or with 2 mmol/L AOA. Control cells gradually lost incorporated BrdUrd with each doubling. In AOA-treated cells, however, the number of BrdUrd-positive cells remained unchanged for 72 hours, 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, because continued treatment of SUM149 and SUM159 cells for 10 days induced cell death (Supplementary Fig. SSC and SSD). 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).
with AOA at 0.5 mg/kg body weight per 3 days a week for 4 weeks \((n = 5–10/\text{group})\). There was a significant reduction in tumor growth in mice treated with AOA (Fig. 6D). In contrast with Balb/c nu/nu mice, doses of AOA above 0.5 mg/kg (doses tested, 0.5, 1, 2.5, and 5 mg/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 \(^{13}\text{C}\)-glucose–derived carbons into glutamate and uridine, and a reduction in oxygen metabolism.
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, were highly elevated in breast cancer cell lines compared with normal breast cells. This reliance on alternative sources of energy, in all likelihood, forms the basis for the addiction of the breast cancer cell to glutamine. A second factor contributing to glutamine addiction could be high levels of c-MYC expression in cells overexpressing neuroblastoma (12). If so, it stands to reason that c-MYC addiction could be high levels of c-MYC expression in cells overexpressing cells (13).

Targeting Glutamine Metabolism in Breast Cancer

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 100 mm³, mice were randomized to control (PBS) or AOA (5 mg/kg i.p., daily) treatment (n = 6–8 mice/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 the Tukey procedure for multiple testing corrections. D, left, tumor-bearing MMTV-rTtA-TetO-myc mice were treated with PBS (n = 10) or AOA 0.5 mg/kg (i.p. 3 days/week; n = 5). Treatment was initiated when the tumors reached 150 to 200 mm³ and was continued for 4 weeks. Right, body weight of animals receiving AOA showed no significant decrease compared to vehicle-treated animals. ANOVA: * , P < 0.0001.
homologous protein), is upregulated downstream of ATF4, causes downregulation of the antiapoptotic mitochondrial protein Bcl-2, promoting apoptosis. Second, ATF6 is activated by proteolytic cleavage following translocation to the Golgi (35). IRE1α activates a JNK signaling pathway, at which point human pro–caspase-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 the amine groups 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 BrdUrd 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 phosphocholine 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 (10 mg/kg body weight) for only 2 weeks, we did not observe tumor growth inhibition at 5 mg/kg body weight of AOA. In our hands, larger doses were associated with loss of weight and signs of toxicity after a week, especially severe 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-tTA-TetO-myc transgenic mouse mammary tumors. By taking advantage of the doxycycline inducibility of the gene in this system, we have 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 anti-tumor 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, because preclinical 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.

Disclosure of Potential Conflicts of Interest
V. Stearns reports receiving commercial research grants from Abbvie, Abraxis, Medimmune, Merck, Novartis, and Pfizer. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: P. Korangath, L. Han, C.V. Dang, V. Stearns, S. Sukumar
Development of methodology: P. Korangath, N. Mori, C.V. Dang, S. Sukumar
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): P. Korangath, H. Sadik, N. Mori, F. Wildes, S. Sukumar
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): P. Korangath, W. W. Teo, H. Sadik, N. Mori, S. Bharti, Z. Zhang, H. Tsai, C.V. Dang, V. Stearns, S. Sukumar
Writing, review, and/or revision of the manuscript: P. Korangath, N. Mori, C.M. Huijts, Z. Zhang, C.A. Santa-Maria, H. Tsai, V. Stearns, Z.M. Bhujwalla, S. Sukumar
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): P. Korangath, C.M. Huijts, Z.M. Bhujwalla, S. Sukumar
Study supervision: C.V. Dang, S. Sukumar

Acknowledgments
The authors thank Dr. Lewis Chodosh, University of Pennsylvania for generously providing MMTV-tTA-TetO-myc transgenic mice and Dr. A. Goga for providing the c-MYC overexpression vector. The authors thank Dilirep Unnikrishnan and Srida Hafeez for laboratory assistance.

Grant Support
This research was supported by Department of Defense Center of Excellence grant W81XWH-04-1-0595 (to S. Sukumar); Susan Komen Foundation for the Cure Postdoctoral grant (PDT12321403; to P. Korangath); the Cindy Rosen-cans Fund for Triple-Negative Breast Cancer Research (to V. Stearns), and the SKCCC Core grant P30 CA069973 (to S. Sukumar).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received May 14, 2014; revised March 10, 2015; accepted March 12, 2015; published OnlineFirst March 26, 2015.

References
Targeting Glutamine Metabolism in Breast Cancer

Targeting Glutamine Metabolism in Breast Cancer with Aminooxyacetate

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


Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-14-1200

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2015/03/27/1078-0432.CCR-14-1200.DC1

Cited articles
This article cites 38 articles, 11 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/21/14/3263.full.html#ref-list-1

Citing articles
This article has been cited by 5 HighWire-hosted articles. Access the articles at:
/content/21/14/3263.full.html#related-urls

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.