Published in final edited form as:

Breast Cancer Res Treat. 2020 October ; 183(3): 549–564. doi:10.1007/s10549-020-05801-4.

Lysine Oxidase Exposes a Dependency on the Thioredoxin Antioxidant Pathway in Triple-Negative Breast Cancer Cells

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Abstract

Purpose—Transformed cells are vulnerable to depletion of certain amino acids. Lysine oxidase (LO) catalyzes the oxidative deamination of lysine, resulting in lysine depletion and hydrogen peroxide production. Although LO has broad antitumor activity in preclinical models, the cytotoxic mechanisms of LO are poorly understood.

Methods—Triple (ER/PR/HER2)-negative breast cancer (TNBC) cells were treated with control media, lysine-free media or control media supplemented with LO and examined for cell viability, caspase activation, induction of reactive oxygen species (ROS) and antioxidant signaling. To determine the role of nuclear factor erythroid 2-related factor 2 (NRF2) and thioredoxin reductase-1 (TXNRD1) in LO-induced cell death, NRF2 and TXNRD1 were individually silenced by RNAi. Additionally, the pan-TXNRD inhibitor auranofin was used in combination with LO.

Results—LO activates caspase-independent cell death that is suppressed by necroptosis and ferroptosis inhibitors, which are inactive against lysine depletion, pointing to fundamental

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

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differences between LO and lysine depletion. LO rapidly induces ROS with a return to baseline levels within 24 hours that coincides temporally with induction of TXNRD activity, the rate-limiting enzyme in the thioredoxin antioxidant pathway. ROS induction is required for LO-mediated cell death and NRF2-dependent induction of TXNRD1. Silencing NRF2 or TXNRD1 enhances the cytotoxicity of LO. The pan-TXNRD inhibitor auranofin is synergistic with LO against transformed breast epithelial cells, but not untransformed cells, underscoring the tumor-selectivity of this strategy.

Conclusions—LO exposes a redox vulnerability of TNBC cells to TXNRD inhibition by rendering tumors cells dependent on the thioredoxin antioxidant pathway for survival.

Keywords

oxidative stress; tumor dependency; metabolism; therapeutics

Introduction

Dysregulation of cellular metabolism is a hallmark of many human malignancies. The metabolic differences between normal cells and tumor cells provide opportunities for developing novel therapeutic approaches for cancer [1, 2]. Normal cells are relatively resistant to the restriction of exogenous amino acids, whereas depletion of select amino acids in cancer cells inhibits cell cycle progression, induces cell death *in vitro* and suppresses tumor growth *in vivo* [3–6]. Depletion of amino acids in tumor cells can be achieved by dietary amino acid restriction [7, 8] or by employing amino acid-degrading enzymes as therapeutic drugs [5, 6, 9]. A number of amino acid-degrading enzymes have been developed over the past decade to deplete specific amino acids. L-lysine- α -oxidase (LO) is a flavoprotein that belongs to the family of L-amino acids oxidases (LAOs). LO catalyzes the irreversible oxidative deamination of the essential amino acid L-lysine (L-Lys), resulting in the production of hydrogen peroxide, ammonia and α -keto- ϵ -aminocaproic acid [10]. Several studies have demonstrated antitumor effects of LO in cultured cancer cells and in xenograft models [11–15]. The antitumor effects of LO have been linked to L-Lys depletion [15] and to the production of hydrogen peroxide and resulting oxidative stress [10, 13]. However, the relative contribution of lysine depletion and oxidative stress to the cytotoxicity of LO, as well as the underlying mechanisms, are poorly understood.

Intriguingly, deficiency of certain amino acids leads to metabolic reprogramming of cancer cells to maintain their increased biosynthetic and energy demands required for tumorigenesis [1, 16–23]. These metabolic changes include activation of a number of pro-survival pathways, which can be therapeutically targeted to enhance cell death in response to amino acid restriction. We have recently developed a new therapeutic paradigm to metabolically prime cancer cells to pro-apoptotic therapy using methionine restriction [24]. In particular, our previous preclinical studies indicate that a dietary restriction of the essential amino acid methionine selectively primes transformed cells to respond to targeted therapy. For example, methionine restriction enhances the cell surface expression of the pro-apoptotic TRAIL receptor-2 and sensitizes triple-negative breast cancer (TNBC) cells, but not untransformed breast epithelial cells, to TRAIL receptor-2 agonists [19]. Moreover, methionine restriction increases expression of MAT2A, the enzyme that converts methionine to S-

adenosylmethionine, and cancer stem cells (CSCs) are highly vulnerable to the combination of methionine depletion and MAT2A inhibition [25]. Based on these findings, we postulate that depletion of other amino acids will also expose metabolic vulnerabilities that can be targeted to enhance cell death.

Here we report that LO activates caspase-independent cell death that is suppressed by inhibitors of necroptosis and ferroptosis, while cell death induced by lysine depletion is unaffected by these inhibitors. LO rapidly and transiently increases reactive oxygen species (ROS) levels and reduces the ratio of reduced/oxidized glutathione in TNBC cells. These markers of oxidative stress return to baseline levels within 24 hours, coinciding temporally with induction of thioredoxin reductase (TXNRD) activity, the rate-limiting enzyme in the cellular thioredoxin antioxidant pathway. ROS induction by LO is required for its cytotoxicity against TNBC cells and leads to the nuclear factor erythroid 2-related factor 2 (NRF2)-dependent induction of TXNRD1 (the cytosolic isoform). Knockdown of NRF2 or TXNRD1 with specific siRNAs induces ROS and enhances the cytotoxicity of LO, pointing to the critical role of these antioxidant pathways in LO-mediated cell death. Indeed, the pan-TXNRD inhibitor auranofin synergistically enhances the cytotoxicity of LO against transformed breast epithelial cells, but not untransformed cells, underscoring the tumor-selectivity of this pro-oxidant combination therapy. Overall, our results indicate that LO exposes a redox vulnerability of TNBC cells to TXNRD inhibition by rendering tumors cells dependent on NRF2-mediated TXNRD1 induction for cell survival.

Material and Methods

Cell culture and reagents

Human MDA-MB-231 TNBC cells stably expressing mCherry and GILM2 TNBC cells were grown as described previously [26]. Human MCF-10A breast epithelial cells stably expressing H-RasV12 or empty vector were cultured as described previously [27]. Cell lines were authenticated by short tandem repeat analysis and tested for mycoplasma contamination prior to experiments. Wild-type (WT) and NRF2 knock out (KO) mouse embryonic fibroblasts (MEFs) were kindly provided by Nobunao Wakabayashi (Fred Hutchinson Cancer Research Center) and grown as described [28]. For lysine deprivation experiments, control medium was formulated by supplementing RPMI-1640 medium (Thermo Fisher Scientific, Waltham, MA) with additional nutrients to closely match the original media for each cell line. Lysine-free media (0% Lys) was formulated in the same way as control media, without the addition of L-lysine. Lysine oxidase (LO), auranofin, buthionine sulfoximine (BSO), and N-acetylcysteine (NAC) were purchased from Sigma-Aldrich (St. Louis, MO). Necrostatin-1 (Necr-1), ferrostatin-1 (Fer-1), and z-VAD-fmk were purchased from Cell Signaling Technology (Beverly, MA).

Crystal violet assay

Cells (3×10^5 cells/well) were seeded on 6-well plates. The next day, cells were washed with PBS and placed in control media, lysine-free media or control media containing LO (10–50 ng/ml). Cells were incubated for 24–72 hours and treated with vehicle, auranofin (0.5 μ M), BSO (20 μ M), NAC (2 mM), necrostatin-1 (Necr-1, 10 μ M), ferrostatin-1 (Fer-1,

10 μ M), zVAD-fmk (10 μ M) or combinations of these agents for the final 24 hours. Surviving cells were stained with crystal violet as described [29].

Trypan blue exclusion assay

Cells (5×10^4 cells/well) were seeded on 24-well plates in triplicate for each assay. The following day, the cells were washed in PBS and cultured in control media, lysine-free media or control media containing LO (13–25 ng/ml) for 0–72 hours. Cells were harvested at each time point and the number of viable cells was scored by Trypan blue (Thermo Fisher Scientific) exclusion.

Cell viability assay

Cell viability was determined by Cell Titer Glo II assay (Promega, Madison, WI, USA). Cells (5×10^3 cells/well) were plated into white-colored 96-well plates. The next day, cells were treated with LO (0–50 ng/ml). Cell viability was measured after 24 hours and expressed as percentage normalized to vehicle-treated cells.

BrdU assay

Cells (2.5×10^3 cells/well in triplicate) were seeded on 96-well plates and allowed to attach overnight. The next day, the medium was changed, and the cells were cultured in control media, lysine-free media or control media with LO (13–25 ng/ml) for 24 hours. BrdU labeling was performed without removing treatment media using the BrdU Cell Proliferation Assay Kit (Cell Signaling Technology, Danvers, MA) according the manufacturer's protocol. BrdU incorporation was detected using a BrdU primary antibody followed by an HRP-linked secondary Ab, and the resulting signal was read in a microplate reader at 450 nm absorbance.

Western blotting

Proteins were immunoblotted from whole-cell lysates using RIPA buffer with a cocktail of protease inhibitors (Sigma-Aldrich) as described [30]. Primary Abs against TXNRD1 (Cell Signaling Technology #15140S), procaspase-3 (Cell Signaling Technology #9662S), p-NRF2^{S40} (Abcam, Cambridge, MA #ab76026) and β -actin (Sigma-Aldrich #A5441) were used.

Cellular ROS assay

Cells (2.5×10^3 cells/well) were seeded in 96-well plates overnight. The next day, media was replaced with control media with or without LO (20 ng/ml) and cells were incubated for 0–72 hours. ROS levels were determined using the DCFDA Cellular ROS Assay kit (Abcam) according to the manufacturer's instructions and expressed as the percentage at the 0 time point.

Reduced glutathione (GSH)/oxidized glutathione (GSSG) assay

Cells were seeded overnight in 100 mm plates (5×10^6 cells/plate). The next day, the media was replaced with control media with or without LO (20 ng/ml) for 0–72 hours. Cells ($1 \times$

10⁶) were lysed and the ratio of GSH/GSSG was determined using the Quantification kit for GSSG and GSH (Sigma-Aldrich) according to the manufacturer's protocol.

Thioredoxin reductase assay

Cells were seeded in 100 mm plates (5×10^6 cells/plate). The following day, the media was replaced with control media with or without LO (20 ng/ml) for 0–72 hours. TXNRD activity was measured in cell lysates using the Thioredoxin Reductase (TXNRD) Assay Kit (Sigma-Aldrich) according to the manufacturer's instructions.

Caspase-3/7 activity assay

Caspase-3/7 activity was measured using the Caspase-Glo 3/7 Assay System (Promega, Madison, WI) according to the manufacturer's instructions. Cells (2.5×10^3 cells/well) were seeded in 96-well plates overnight. The following day, media was replaced with control media, lysine-free medium or control media containing LO (20 or 50 ng/ml). Cells were incubated for 24 hours and treated with vehicle, auranofin (0.5 μ M) or combination of these agents for the final 5 hours. Caspase-3/7 activity (expressed as fold activity compared to controls) was measured by luminescence using a plate reader and was normalized to cell confluence.

Real-time PCR

cDNA was first synthesized from total RNA (iScript™ cDNA Synthesis Kit, Bio-Rad, Hercules, CA). cDNA was then PCR amplified using the iQ™ SYBR Green supermix (Bio-Rad) and the following primers (Integrated DNA Technologies): NRF2 (forward 5-GAGCAAGTTTGGGAGGAGCT-3, reverse 5-GGTTGGGGTCTTCTGTGAG-3) human TXNRD1 (forward 5-GCTTTCACGTACTGGGTCCA-3, reverse 5-TGCACAGACAGGGTGGATTC-3) or GAPDH (5-GAAGGTGAAGGTCGGAGTC-3, reverse 5-GAAGATGGTGATGGGATTTC-3). The products were detected using the CFX96 Real Time PCR sequence detection system (Bio-Rad). RNA levels were normalized to control (GAPDH) levels in each experiment using a comparative Ct method. Each analysis was performed twice in triplicate.

NRF2 and TXNRD1 siRNA experiments

ON-TARGETplus siRNAs targeting human NRF2 or human TXNRD1 and non-silencing control siRNAs were purchased from Dharmacon/Horizon Discovery (Lafayette, CO). Cells were transfected with siRNAs using Lipofectamine RNAiMAX Reagent (Invitrogen) according to the manufacturer's instructions.

Statistical analysis

ANOVAs with Tukey's multiple comparison test were done using Prism 6 GraphPad Software (San Diego, CA) to assess statistical significance.

Results

Lysine oxidase is more cytotoxic than lysine depletion

To compare the cytotoxic effects of LO and lysine deprivation, MDA-MB-231 and GILM2 TNBC cells were cultured in control media, lysine-free media or control media supplemented with LO for 72 hours. Both treatments inhibited growth of MDA-MB-231 and GILM2 cells as determined by trypan blue exclusion, but LO inhibited cell growth more robustly than lysine depletion at the end of experiment in both TNBC cell lines (Fig. 1A). Furthermore, both lysine restriction and LO inhibited cell proliferation as determined by BrdU incorporation (Fig. 1B). LO was more cytotoxic against TNBC cells than lysine restriction, particularly at higher concentrations of LO (Fig. 1C). Dose-response experiments identified LO IC₅₀s of approximately 10 and 14 ng/ml for MDA-MB-231 and GILM2 cells, respectively (Fig. 1D). Taken together, these findings indicate that both lysine deprivation and LO inhibit cell proliferation and reduce cell viability, although LO is more cytotoxic against TNBC cells than lysine depletion.

LO activates caspase-independent necroptosis and ferroptosis cell death pathways

We next examined the mechanisms by which LO induces cell death. Neither lysine depletion or LO increased caspase-3/7 activity in MDA-MB-231 and GILM2 cells (Fig. 2A) or induced proteolytic processing of procaspase-3 as detected by diminished procaspase-3 expression (Fig. 2B). These findings indicate that LO and lysine deprivation do not induce caspase-dependent apoptotic cell death in TNBC cells. Consistent with this observation, the broad-spectrum caspase inhibitor zVAD-fmk did not inhibit the cytotoxicity of lysine deprivation or LO in these TNBC cells (Figs. 2C and 2D). In contrast, both the necroptosis inhibitor necrostatin-1 (Ncr-1) [31] and the ferroptosis inhibitor ferrostatin-1 (Fer-1) [32] attenuated the cytotoxicity of LO but not lysine depletion. Collectively, these results indicate that LO induces caspase-independent necroptosis and ferroptosis and triggers cell death by a mechanism distinct from that of lysine depletion.

The cytotoxicity of LO is dependent on ROS production

Since the degradation of lysine by LO produces hydrogen peroxide [5], we measured ROS levels in MDA-MB-231 and GILM2 TNBC cells treated with LO. Strikingly, LO rapidly increased ROS levels within 1 hour of treatment, with levels peaking at 3–6 hours and returning to baseline within 24 hours (Fig. 3A and 3B). This transient induction of oxidative stress was accompanied by a corresponding reduction in the ratio of reduced to oxidized glutathione ratio (GSH/GSSG) from 1–12 hours, with a return to baseline by 24 hours. In addition, the activity of the cellular antioxidant enzyme thioredoxin reductase, which catalyzes the rate-limiting step in the thioredoxin antioxidant pathway (33), was increased in response to LO treatment and remained elevated for 48–72 hours. To investigate whether ROS induction is required for the cytotoxic activity of LO, MDA-MB-231 and GILM2 cells were incubated with LO in the absence or presence of the ROS scavenger, N-acetyl cysteine (NAC). NAC markedly inhibited the cytotoxicity of LO (Fig. 3C) and ROS induction by LO (Fig. 3D) in both TNBC cell lines. Taken together, the results indicate that (1) LO initiates oxidative stress and activates the cellular TXNRD antioxidant pathway, and (2) the cytotoxicity of LO is ROS-dependent.

LO induces TXNRD1 by a ROS- and NRF2-dependent mechanism

To investigate the mechanisms by which LO activates the TXNRD antioxidant pathway, we postulated that LO-mediated oxidative stress would induce NRF2, the master transcriptional regulator of the antioxidant response [34]. Indeed, p-NRF2 levels were rapidly and transiently increased in MDA-MB-231 and GILM2 cells treated with LO (Fig. 4A) with a similar time frame as ROS induction (Fig. 3A and 3B). The NRF2 transcriptional target TXNRD1 (cytosolic TXNRD isoform) [33] was also rapidly induced by LO treatment and levels remained elevated for the 72 hour duration of the experiment. Notably, the induction of both p-NRF2 and TXNRD1 by LO was inhibited by NAC, underscoring that their induction by LO is ROS-dependent (Fig. 4B). To examine whether the induction of TXNRD1 by LO is mediated by NRF2, we treated WT and NRF2^{-/-} MEFs with LO and observed that NRF2 deletion suppressed the induction of TXNRD1 protein levels (Fig. 4C) and TXNRD activity (Fig. 4D) by LO. These results indicate that LO regulates TXNRD1 expression by a ROS- and NRF2-dependent mechanism.

Silencing NRF2 enhances the cytotoxicity of LO

To examine the functional role of NRF2 induction in LO-mediated cell death, MDA-MB-231 and GILM2 cells were transfected with scrambled control siRNAs (siC) or one of two different siRNAs targeting NRF2 (si1 NRF2 and si2 NRF2). Both siRNAs targeting NRF2 robustly reduced NRF2 mRNA levels compared to the scrambled control siRNA (Fig. 5A). Silencing NRF2 in both TNBC cell lines induced ROS (Fig. S1) and inhibited the increase in TXNRD activity in response to LO treatment (Fig. 5B). Moreover, silencing NRF2 robustly sensitized TNBC cells to LO treatment compared to a scrambled control siRNA (Fig. 5C and 5D). These findings indicate that NRF2 induction by LO promotes cell survival in the setting of LO-mediated oxidative stress.

Silencing TXNRD1 augments the cytotoxicity of LO

To determine the specific role of TXNRD1 induction in LO-mediated cell death, MDA-MB-231 and GILM2 cells were transfected with scrambled control siRNAs (siC) or one of two different siRNAs targeting TXNRD1 (si1 TXNRD1 and si2 TXNRD1). Silencing TXNRD1 markedly reduced TXNRD1 mRNA levels (Fig. 6A) and TXNRD activity (Fig. 6B) and induced ROS (Fig. S1) in TNBC cells. Additionally, silencing TXNRD1 markedly increased the cytotoxicity of LO in TNBC cells (Fig. 6C and 6D). These findings point to TXNRD1 as a redox vulnerability of TNBC cells exposed to LO.

The TXNRD inhibitor auranofin enhances the cytotoxicity of LO by a ROS-dependent mechanism

Given our observation that TXNRD1 silencing was synthetic lethal in combination with LO, we treated TNBC cells with the pan-TXNRD inhibitor auranofin [35] alone or in combination with LO. Auranofin, but not buthionine sulfoximine (BSO), an inhibitor of glutathione biosynthesis (36), markedly sensitized TNBC cells to LO-induced cell death (Fig. 7A and 7B). Synergy analyses by Excess over Bliss methodology revealed that the combination of LO and auranofin was synergistic at multiple LO doses (Fig. S2). The effects of auranofin were reversed by NAC (Fig. 7C), indicating that the ability of auranofin to

sensitize TNBC cells to LO is ROS-dependent. Furthermore, both necrostatin-1 (Necr-1) and ferrostatin-1 (Fer-1) attenuated cell death induction by auranofin and LO, while zVAD had no effect (Fig. 7D). Consistent with these findings, auranofin, LO or the combination did not increase caspase-3/7 activity (Fig. S3). The results indicate that auranofin enhances LO-induced necroptosis and ferroptosis by a ROS-dependent mechanism.

Transformed breast epithelial cells are more sensitive to LO than non-transformed cells

To determine whether transformed breast epithelial cells are more susceptible to lysine depletion or LO, we cultured MCF-10A breast epithelial cells transformed by oncogenic H-RasV12 or non-transformed MCF-10A expressing empty vector cells in control media, lysine-free media or control media with LO. Transformed MCF-10A-Ras cells were moderately more sensitive to lysine depletion but markedly more susceptible to LO (Fig. 8A). Moreover, transformed MCF-10A-Ras cells were also more sensitive to the combination of LO and auranofin (Fig. 8B). Taken together, these findings indicate that Ras-transformed cells are more vulnerable to LO or the combination of LO and auranofin than non-transformed cells, suggesting a favorable therapeutic index for this combination therapy.

Discussion

Due to its broad cytotoxic activity in cellular and murine models of cancer (11–15), LO has garnered interest as a cancer therapy. The cytotoxicity of LO has been attributed to extracellular lysine degradation [15] and hydrogen peroxide production, the latter resulting in oxidative stress [10, 13]. Consistent with these findings, we observed that both lysine depletion and LO inhibit proliferation and reduce cell viability of TNBC cells, but LO is more cytotoxic than lysine depletion. Our *in vitro* results are congruent with the reported modest effects of lysine depletion on tumor growth *in vivo* [37]. Although both lysine deprivation and LO do not activate caspases, we have demonstrated that the cytotoxic mechanisms of lysine depletion and LO are fundamentally different. Cell death induced by LO, but not lysine depletion, is suppressed by necrostatin-1 and ferrostatin-1, pointing to necroptosis and ferroptosis as the principal cell death mechanisms initiated by LO. Moreover, LO rapidly initiates oxidative stress within 1 hour of treatment as determined by increased ROS levels and reduced GSH/GSSG ratio. Notably, the observed induction of oxidative stress by LO is required for its cytotoxicity as NAC robustly inhibits LO-mediated cell death. These latter findings are consistent with previous reports that catalase inhibits LO-induced cell death [15]. Strikingly, the oxidative stress induced by LO is transient and is reversed within 24 hours of treatment, strongly suggesting that cellular antioxidant pathways are activated by LO treatment to enable cell survival.

Here we report for the first time that the thioredoxin reductase antioxidant pathway and its rate-limiting enzyme TXNRD1 [33] are activated by LO, thereby exposing a targetable redox vulnerability. Several lines of evidence support this conclusion. First, the master antioxidant transcriptional regulator NRF2 [34], TXNRD1 and TXNRD activity are rapidly induced by LO in parallel to ROS induction. Second, the induction of TXNRD1 by LO is dependent on (1) NRF2, as determined by loss of induction in NRF2-deficient MEFs and (2) ROS, as the induction of NRF2 and TXNRD1 is suppressed by NAC. Third, silencing NRF2

or TXNRD1 inhibits TXNRD activity, induces ROS and markedly enhances LO-induced cell death consistent with a lethal response to overwhelming oxidative stress. In contrast, inhibition of glutathione biosynthesis with BSO has no effect on LO-mediated cell death, pointing to the specific dependence of cancer cells treated with LO on the thioredoxin antioxidant pathway. Overall, these results point to the NRF2-TXNRD1 cellular antioxidant response as a key mediator of cell survival in response to LO treatment and a targetable redox vulnerability unmasked by LO.

To exploit this metabolic liability, we combined LO with the pan-TXNRD inhibitor auranofin, an FDA-approved antirheumatic agent [35]. Auranofin has emerged as a promising cancer therapeutic agent when used in combination with other pro-oxidant agents such as glutathione inhibitors [38, 39]. As predicted, auranofin, but not the glutathione biosynthesis inhibitor BSO, synergistically augmented the cytotoxicity of LO against TNBC cells. These effects were ROS-dependent as NAC suppressed cell death sensitization by auranofin. Consistent with our observations regarding LO-mediated cell death, both necrostatin-1 and ferrostatin-1 (but not z-VAD-fmk) inhibited cell death induction by the combination of LO and auranofin. Moreover, breast epithelial cells transformed by RasV12 were more sensitive to LO or the combination of LO and auranofin than non-transformed breast epithelial. These latter results point to a degree of tumor-selectivity of the combination of LO and auranofin that likely reflects the enhanced vulnerability of transformed cells to oxidative stress.

In summary, we have demonstrated that LO exposes a targetable metabolic vulnerability of TNBC cells to TXNRD inhibition by ROS- and NRF2-dependent induction of TXNRD1. These results are consistent with our therapeutic model of “metabolic priming” whereby we use nutritional deficiencies to unmask metabolic vulnerabilities that can be targeted to enhance therapeutic response [24]. For example, we have demonstrated that restriction of the essential amino acid methionine primes tumors to respond to TRAIL receptor-2 agonists by increasing cell surface expression of TRAIL receptor-2 [19]. More recently, we showed that methionine restriction sensitizes cancer stem cells to MAT2A inhibition by increasing expression of MAT2A, the enzyme that synthesizes S-adenosylmethionine from methionine [25]. As LO has been shown to inhibit tumor growth *in vivo* and is well tolerated in mice [11–15], we plan to examine its antitumor effects in combination with auranofin in murine models in future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We are indebted to Nobunao Wakabayashi (Fred Hutchinson Cancer Research Center) for providing NRF2^{-/-} MEFs used in these studies.

Funding

This work was supported by grants from the V Foundation for Cancer Research (VLC), Breast Cancer Research Foundation (VLC), Wisconsin Partnership Program, and National Institutes of Health University of Wisconsin

Comprehensive Cancer Center P30CA14520 core facility support, the Russian Academic Excellence Project 5-100 (AZ).

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Fig. 1. Lysine oxidase is more cytotoxic than lysine restriction.

A, MDA-MB-231 and GILM2 cells were grown in control media, lysine-free media or control media supplemented with LO (25 ng/ml for MDA-MB-231 cells or 13 ng/ml for GILM2 cells) for the indicated number of hours, and the number of viable cells were scored by trypan blue exclusion. The data are presented as fold cell number compared to cells in control media (mean $\pm$ SEM, $n = 3$). B, MDA-MB-231 and GILM2 cells were grown in control media, lysine-free media or control media supplemented with LO (25 ng/ml for MDA-MB-231 cells or 13 ng/ml for GILM2 cells) for 24 hours and the percentage of BrdU-

positive cells was determined (mean $\pm$ SEM, $n = 3$). **C**, Crystal violet staining of MDA-MB-231 and GILM2 TNBC cells grown in control media, lysine-free media or control media supplemented with LO (25 or 50 ng/ml for MDA-MB-231 cells and 10 or 40 ng/ml for GILM2 cells) for 24 hours. Top panel: representative images. Bottom panel: quantification performed by scoring cell confluence in 3 fields of each well (mean $\pm$ SEM, $n = 3$). **D**, CellTiter Glo cell viability assay of GILM2 and MDA-MB-231 TNBC cells treated with LO (0–50 ng/ml) for 24 hours. Cell viability is expressed as the percentage normalized to vehicle-treated cells ($n=3$). In all panels, *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$ versus control or the indicated comparison.

Fig. 2. LO activates caspase-independent necroptosis and ferroptosis cell death pathways.

A, Caspase-3/7 activity of MDA-MB-231 and GILM2 cells cultured for 24 hours in control media, lysine-free media or control media supplemented with LO (20 or 50 ng/ml) for the final 5 hours. The data are presented as fold activity compared to cells grown in control media (mean $\pm$ SEM, $n = 2$). **B**, Immunoblot of procaspase-3 expression in MDA-MB-231 and GILM2 cells grown in complete media supplemented with LO (20 ng/ml) for 24 hours. **C** and **D**, Crystal violet cell survival assay of MDA-MB-231 (**C**) and GILM2 (**D**) cells grown in control medium, lysine-free media or control media supplemented with LO (50

ng/ml) in the presence or absence of necrostatin-1 (Ncr-1, 10 μ M), ferrostatin-1 (Fer-1, 10 μ M) or z-VAD-fmk (10 μ M) for 24 hours. Left panel: representative images. Right panel: quantification performed by scoring cell confluence in 3 fields of each well (mean $\pm$ SEM, n = 3). In (**C**) and (**D**), **, $P < 0.01$, ***, $P < 0.001$ versus vehicle control.

Fig. 3. The cytotoxicity of LO is dependent on ROS production.

A and **B**, MDA-MB-231 (**A**) and GILM2 (**B**) TNBC cells cultured in control media supplemented with LO (20 ng/ml) for the indicated number of hours. ROS levels (percentage at time $t=0$), GSH/GSSG ratio, and TXNRD activity were measured (mean $\pm$ SEM, $n = 3$). **C**, Crystal violet cell survival assay of MDA-MB-231 and GILM2 cells grown in control media supplemented with LO (50 ng/ml) in the presence or absence of N-acetylcysteine (NAC, 2 mM) for 24 hours. Top panel: representative images. Bottom panel: quantification performed by scoring cell confluence in 3 fields of each well (mean $\pm$ SEM, $n = 3$). **D**,

MDA-MB-231 and GILM2 cells grown in control media supplemented with LO (20 ng/ml) in the presence or absence of NAC (2 mM) for 6 hours. ROS levels are expressed as percentage of vehicle-treated cells. In all panels, *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$ versus control or the indicated comparisons.

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Fig. 4. LO induces TXNRD1 by a ROS- and NRF2-dependent mechanism.

A, Immunoblot of p-NRF2, TXNRD1 expression in MDA-MB-231 and GILM2 cells cultured in control media supplemented with LO (20 ng/ml) for the indicated number of hours. **B**, Immunoblot of p-NRF2 and TXNRD1 expression in MDA-MB-231 and GILM2 cells grown in control media or control media supplemented with LO (20 ng/ml) in the presence or absence of NAC (2 mM) for 12 hours. **C**, Immunoblot of p-NRF2 and TXNRD1 expression in WT MEFs and NRF2^{-/-} MEFs cultured in control media with or without LO (20 ng/ml) for 48 hours. **D**, TXNRD activity (percent control) in WT MEFs and NRF2^{-/-} MEFs cultured in control media with or without LO (20 ng/ml) for 48 hours (mean $\pm$ SEM, n = 3). In **(D)**, ***, $P < 0.001$ versus control.

Fig. 5. Silencing NRF2 enhances the cytotoxicity of LO.

A MDA-MB-231 and GILM2 cells were transfected with siRNAs targeting NRF2 (si1 NRF2 or si2 NRF2) or non-silencing control siRNAs (siC), and NRF2 mRNA levels were measured by RT-PCR 48 hours after transfection. NRF2 mRNA levels are presented as fold change compared to levels in control siRNA-transfected cells. **B**, TXNRD activity of MDA-MB-231 and GILM2 cells transfected with control (siC) or NRF2 siRNAs (si1 NRF2, si2 NRF2) and cultured in control media in presence or absence of LO (20 ng/ml) for 48 hours. **C** and **D**, Crystal violet cell survival assay of MDA-MB-231 (**C**) and GILM2 (**D**) cell lines

transfected with control (siC) or NRF2 siRNAs (si1 NRF2, si2 NRF2) and cultured in the presence or absence of LO (20 ng/ml) for 48 hours. Left panel: representative images. Right panel: quantification performed by scoring cell confluence in 3 fields of each well (mean $\pm$ SEM, n = 3). In all panels, **, $P < 0.01$, ***, $P < 0.001$ versus control.

Fig. 6. Silencing TXNRD1 augments the cytotoxicity of LO.

A, MDA-MB-231 and GILM2 cells were transfected with siRNAs targeting TXNRD1 (si1 TXNRD1 or si2 TXNRD1) or non-silencing control siRNAs (siC), and TXNRD1 mRNA levels were determined by RT-PCR 48 hours after transfection. TXNRD1 mRNA levels are presented as fold change compared to levels in control siRNA-transfected cells. **B**, TXNRD activity of MDA-MB-231 and GILM2 cells transfected with non-silencing control (siC) siRNAs or TXNRD1 siRNAs (si1 TXNRD1 or si2 TXNRD1). **C** and **D**, Crystal violet cell survival assay of MDA-MB-231 (**C**) and GILM2 (**D**) cells transfected with control (siC) or

TXNRD1 siRNAs (si1 TXNRD1 or si2 TXNRD1) and cultured in control media in the presence or absence of LO (20 ng/ml) for 48 hours. Left panel: representative images. Right panel: quantification performed by scoring cell confluence in 3 fields of each well (mean $\pm$ SEM, n = 3). In all panels, ***, $P < 0.001$ versus control.

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Fig. 7. The TXNRD inhibitor auranofin enhances the cytotoxicity of LO by a ROS-dependent mechanism.

A and B, Crystal violet cell survival assay of MDA-MB-231 (**A**) and GILM2 (**B**) cells cultured in control media or control media supplemented with LO (20 ng/ml) in the presence or absence of auranofin (0.5 μ M) or BSO (20 μ M) for 48 hours. Top panel: representative images. Bottom panel: quantification performed by scoring cell confluence in 3 fields of each well (mean $\pm$ SEM, $n = 3$). **C,** Crystal violet cell survival assay of MDA-MB-231 and GILM2 cells cultured in control media or control media supplemented with of LO (20

ng/ml) in the presence or absence of auranofin (0.5 μ M) or NAC (2 mM) for 48 hours (mean $\pm$ SEM, n = 3). **D**, Crystal violet cell survival assay of MDA-MB-231 and GILM2 cell lines grown in control media or control media with LO (20 ng/ml), in the presence or absence of auranofin (0.5 μ M), necrostatin-1 (Ncr-1, 10 μ M), ferrostatin-1 (Fer-1, 10 μ M) or z-VAD-fmk (10 μ M) for 48 hours (mean $\pm$ SEM, n = 3). In all panels, *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$ versus control or the indicated comparisons.

Fig. 8. Transformed breast epithelial cells are more sensitive to LO than non-transformed breast epithelial cells.

A, Crystal violet cell survival assay of MCF-10A breast epithelial cells stably expressing empty vector (MCF-10A-Vector) or oncogenic H-RasV12 (MCF-10A-RAS) grown in control media, lysine-free media, or control media supplemented with LO (50 ng/ml) for 48 hours. Top panel: representative images. Bottom panel: quantification performed by scoring cell confluence in 3 fields of each well (mean $\pm$ SEM, $n = 3$). **B**, Crystal violet cell survival assay of MCF-10A-Vector and MCF-10A-Ras cells grown in control media or control media

supplemented with LO (50 ng/ml for MCF-10A-Vector cells and 20 ng/ml for MCF-10A-RAS cells) in the presence or absence auranofin (0.5 μ M) for 48 hours. Top panel: representative images. Bottom panel: quantification performed by cell confluence in 3 fields of each well (mean $\pm$ SEM, n = 3). In all panels, *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$ versus control or the indicated comparison.

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