α -Linolenic Acid Induces Breast Cancer Cell Apoptosis by Down-Regulating Human Fatty Acid Synthase

Wenyuan Huang

University of Chinese Academy of Sciences School of Computer and Control Engineering; University of the Chinese Academy of Sciences School of Computer Science and Technology

Xing Guo

University of the Chinese Academy of Sciences

Huijin Fan

University of Chinese Academy of Sciences

Amantay Alzhan

University of the Chinese Academy of Sciences

Zhengan Liu

Institute of Botany Chinese Academy of Sciences

Xiaofeng Ma (✉ maxiaofeng@ucas.ac.cn)

University of the Chinese Academy of Sciences <https://orcid.org/0000-0001-9834-5477>

Qingyan Shu

Institute of Botany Chinese Academy of Sciences

Research Article

Keywords: Fatty acid synthase, α -Linolenic acid, Inhibitor, Breast cancer cells, Cell apoptosis.

Posted Date: April 5th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-344421/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

α -Linolenic acid induces breast cancer cell apoptosis by down-regulating
human fatty acid synthase

Wenyuan Huang^a

huangwenyuan16@mails.ucas.ac.cn

Xing Guo^a

guoxing19@mails.ucas.ac.cn

Huijin Fan^b

fanhuijin19@mails.ucas.ac.cn

Amantay Alzhan^a

alzhan.zh@mail.ru

Zhengan Liu^c

liuzhengan@ibcas.ac.cn

Xiaofeng Ma^{a,*}

maxiaofeng@ucas.ac.cn

Qingyan Shu^{c,*}

shuqy@ibcas.ac.cn

^a College of Life Sciences, University of Chinese Academy of Sciences, No. 19A Yuquan Road, Beijing
100049, China

^b Medical College, University of Chinese Academy of Sciences, No. 19A Yuquan Road, Beijing 100049,
China

^c Key Laboratory of Plant Resources and Beijing Botanical Garden, Institute of Botany, the Chinese
Academy of Sciences, Beijing 100093, China

* Corresponding author.

Qingyan Shu, Ph. D, Associate Professor, Key Laboratory of Plant Resources and Beijing Botanical
Garden, Institute of Botany, the Chinese Academy of Sciences, Beijing 100093, China; Email:
shuqy@ibcas.ac.cn; Tel. /Fax: +8610-62836655

Xiaofeng Ma, Ph. D, Associate Professor, College of Life Sciences, University of Chinese Academy of
Sciences, No. 19A Yuquan Road, Beijing 100049, China; Email: maxiaofeng@ucas.ac.cn; Tel. /Fax:
+8610-88256585

Abstract

Background/Aims: Fatty acid synthase (FASN) is a multi-enzyme complex that synthesizes endogenous fatty acids. The overexpression of FASN has been reported in variety of cancers, including breast, ovarian, prostate, and colon cancers, which strongly indicates the involvement of FASN in cancer progression. α -Linolenic acid (ALA) has many biological activities, including anti-cancer effects. The aim of the present study was to investigate the inhibitory effect of ALA on fatty acid synthesis pathway and apoptosis of human breast cancer cells.

Methods: Cytotoxicity of ALA in human breast cancer cells (MDA-MB-231 and MCF-7 cells) was assessed by MTT assay. Changes in protein expression were detected by immunoblot analysis. Cell migration was detected by wound healing and cell transwell assay. Apoptotic effects, mitochondrial membrane potential and cell cycle analysis were detected by flow cytometry. Molecular docking was carried out with the AutoDockTools program suite to analyze the binding abilities of ALA on thioesterase (TE) domain of FASN.

Results: We found that the expression levels of FASN decreased significantly in ALA treated breast cancer cells. Compared with palmitic acid (PA), ALA reduced cell viability in a dose-dependent manner. ALA showed a higher affinity with the TE domain than PA. ALA induced breast cancer cells apoptosis, which effects were similar with the knockdown of FASN. In addition, ALA inhibited the invasion and metastasis, and arrested cell cycle in breast cancer cells.

Conclusion: We propose a hypothesis that ALA could contribute to the treatment of human breast cancer by inhibiting FASN.

Key words: Fatty acid synthase; α -Linolenic acid; Inhibitor; Breast cancer cells; Cell apoptosis.

Background

Breast cancer is one of the most serious diseases to threaten female's health [1]. Although some of them who suffered from breast cancer could be cured, however, there are lots of people has poor prognosis which leading to death [2]. It has high risk to invade other tissues and organs. Breast cancer invasion and metastasis are the main reasons to cause of patient's death [3, 4]. Until now, plenty of drugs have been developed to treat breast cancer, however breast cancer remains a complex health problem for woman around the world [5]. Therefore, development of effective drug to treat breast cancer is an urgent need.

Many researches have explored that fatty acid synthase (FASN, EC 2.1.3.85) expression in breast cancer cell various from normal to overexpressed in cancer cell [6, 7]. High levels of FASN expression and activity provide favorable conditions for tumor growth and progression [8, 9]. FASN is considered as a marker of poor prognosis [10]. FASN is an enzyme that catalyzes the synthesis of long-chain fatty acid using acetyl-coenzyme A (CoA), malonyl-CoA, and NADPH as its substrates [9]. As the only enzyme that endogenously synthesizes fatty acids in cells, FASN plays a vital role in cell growth. Fatty acids are irreplaceable factors in cell growth and important parts of cell membranes. In addition, some intermediate fatty acids metabolites could directly stimulate cell growth [11]. Previous study revealed that 93% of the fatty acids which consumed by cancer cells are catalyzed by FASN, meanwhile the main fatty acids which used by normal cells come from diets [12]. Since FASN plays an essential role in breast cancer and it is totally contrasting between normal cells and cancer cells, FASN is considered as a new target. It has been identified that inhibiting FASN could induce cancer cell apoptosis, but the mechanism remains to be studied [13, 14]. Palmitic acid (PA), the main product of FASN catalyzed reaction, is reduced after inhibition of FASN, which then leads to apoptosis of cancer cells [15]. Malonyl-CoA will be accumulated after the suppression of FASN. Excess malonyl-CoA will repress acylcarnitine transferase 1, and the apoptosis pathway will be triggered [16]. Many studies have illustrated the relationship between FASN and

molecular pathway signaling, such as P53 pathway PI3K-AKT and MAPK etc. [17, 18]. Therefore, the discovery of FASN inhibitors would bring new opportunity for metabolically combating cancer.

α -Linolenic acid (ALA) is a long chain fatty acid with 18 carbon and three *cis* double bonds. Most of the ALA comes from plant seeds, especially from flax and peony seeds. ALA is an essential fatty acid for human being but cannot be synthesized by the human body [19]. ALA assists human to keep health by direct and indirect way. If there is a lack of ALA in the diet, the person may suffer from chronic diseases such as obesity, atherosclerosis and cardiovascular disease [20, 21]. ALA can improve leptin levels and decrease epididymal adiposity, as well as adipocyte size in mice [22]. ALA administration prevents diabetes and its complications via PI3K/AKT signaling [23]. Besides, many studies indicate that ALA exerts anti-inflammatory and antioxidant effects through increasing the expression level of Nrf2 [24, 25]. In addition, ALA transforms other biological substances such as DHA and EPA, as a precursor. These biological molecules could contribute to the metabolism of saturated fatty acids, which reduces the risk of atherosclerosis [26]. Recently, more and more evidence prove that ALA has an anti-cancer effect, but the mechanism remains unknown [27, 28].

Since ALA could reduce adipose tissue size and induce cancer cell apoptosis, meanwhile FASN plays a significant role in fat synthesis and cancer, therefore it is proposed that ALA induces apoptosis of breast cancer cell by inhibiting FASN. To investigate whether ALA-mediated cancer cell apoptosis is related to FASN, firstly, the effect of ALA on FASN expression in breast cancer cells was tested. Cytotoxicity of ALA was explored, subsequently, we detected whether ALA affects breast cancer cell invasion and metastasis. The discovery of ALA as a new FASN inhibitor will provide one of the potential perspective anticancer treatments.

Methods

Reagents

100 ALA, PA, DMSO, and 5-tetradecyloxy-2-furoic acid (TOFA) were purchased from Sigma (St. Louis, MO,
101 USA). RPMI 1640 Medium basic and phosphate-buffered saline (PBS) were purchased from Biological
102 Industries (Israel). Fetal bovine serum (FBS) was purchased from South America sterile filtered. Antibodies of
103 FASN, IRE1, ATF6, PERK, and CHOP were purchased from Cell Signaling Technology (Denvers, MA, USA).
104 GAPDH was purchased from Lablead (Beijing, China).

105 **Cell lines and culture**

106 The human breast cancer cell lines MDA-MB-231 and MCF-7 cells were purchased from the Type Culture
107 Collection of the Chinese Academy of Sciences, Shanghai, China. Cells were grown in DMEM, supplemented
108 with 10% FBS and were maintained in a humidified atmosphere containing 95% air and 5% CO₂ at 37 °C.

109 **Cell viability assay**

110 Cell viability assays were performed in 96-well plates. MDA-MB-231 and MCF-7 cells were cultured in the
111 plates until confluence and were incubated with either DMSO (1:1000) or increasing doses of ALA for 24 h at
112 37 °C. Cell medium was then changed to a fresh one within 500 µg/mL MTT. After 4 h incubation at 37 °C, the
113 plates were decanted and added 150 µL DMSO to solubilize the formazan crystals. The plate was analyzed by
114 a microplate spectrophotometer (Multiskan, MK3) at the wavelength of 492 nm. All experiments were carried
115 out five times on three different occasions.

116 **Analysis of apoptosis**

117 At 24 h after ALA treatment, the cells were collected and washed twice with cold PBS, and then resuspended
118 at a density of 1×10^6 cells per mL. The cell suspension (500 µL) was incubated with Annexin-V-FITC (5 µL)
119 and PI (10 µL) for 15 min in dark, and analyzed with FACScalibur instrument (Becton–Dickinson, San Jose,
120 US) within 1 h.

121 **Western blot analysis**

Following treatment of breast cancer cells with corresponding concentration of ALA for 24 h, cells were collected using trypsin-EDTA and washed twice with PBS. Then, a particle free supernatant was obtained by centrifuging at 12,000 g for 20 min at 4 °C. The protein concentration of cell lysates was measured by Pierce BCA protein analysis kit. Comparable amounts of protein were separated using a 10%-12% SDS-PAGE and transferred to PVDF membranes. Then probed with primary antibodies dilution overnight at 4 °C. After washing thrice with TBST solution, cells were incubated with corresponding secondary antibody for 1 h, and developed with a commercial kit. Western blotting was performed with anti GAPDH antibody to control protein loading and transfer. All experiments were carried out at least three times.

Mitochondrial membrane potential test

The mitochondrial membrane potential ($\Delta\Psi_m$) was detected as described previously [15]. Briefly, after treating with ALA (0, 25, 50, 75, and 100 μ M) for 24 h, cells were digested with trypsin, resuspended at a density of 1×10^6 cells per mL. After washing thrice with cold PBS, the cells were then stained and incubated with 25 μ M JC-1 for 30 min at 37 °C. Finally, the fluorescence shift of JC-1 was measured by flow cytometry to evaluate mitochondrial dysfunction.

Immunofluorescence

The FASN expression and distribution in the cell were investigated by immunofluorescence confocal microscopy, after treating with ALA for 24 h, breast cancer cells were planted in the 24-well plate for 12 h. Cultured cells were exposed with various concentrations of ALA for 24 h and then fixed with 4% paraformaldehyde for 10 min, PBST was used to wash cells twice, permeabilized with 0.1% Triton X-100 and then blocked in 3% BSA for 1 h at room temperature. Before staining cell nucleus by DAPI for 1 h without light, incubation with FASN antibodies dilution at a concentration of 1:100 overnight at 4 °C. After that, the image was analyzed by confocal microscopy.

144 **RNA interference**

145 The FASN-targeted siRNA (TGG AGC GTA TCT GTG AGA ATT) and the negative RNA (UUC UCC GAA
146 CGU GUC ACG UTT) were purchased from GenePharma (Shanghai, China). The siRNA was transfected into
147 cells using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions.

148 **Wound healing**

149 Breast cancer cells were seeded into 6-well plates (2×10^6 cells per well) in DMEM with 10% FBS overnight.
150 Cells were scratched for a straight line by 200 μ L pipette tip and then PBS buffer was used to clean cells for
151 three times. DMEM containing different ALA concentrations was used to treat cells for 24 h. The cell migration
152 was observed by microscope.

153 **Cell migration assay**

154 Transwell assay was applied to measure cell migration. Breast cancer cells were plated in the upper chamber
155 overnight till to an 80% confluency. Then, different concentrations of ALA were added into DMEM to culture
156 cells at upper chamber while DMEM with 10% FBS was used in bottom chamber. After 24 h, the upper chamber
157 cell should be removed, migrated cells which appears on under chamber were stained by crystal violet. The
158 results were shown by microscope.

159 **Cell cycle assay**

160 Cells were seeded in the 6-plates overnight till at a density of 80%. Cells were cleaned three times using PBS
161 buffer and then virous concentrations of ALA were used to treat cells for 24 h. After that, cells were harvested
162 to analysis cell cycle by flow cytometry according to the instruction provided by the manufacturer strictly.

163 **Molecular docking**

164 The occupied position of polyunsaturated fatty acyl adduct in the crystal structure of the human FASN
165 thioesterase (TE) domain (PDB_ID:3TJM) was used as the grid box center ($70 \times 70 \times 70 \text{\AA}$) for docking.

AutoDockTools program suite was used to pretreat the structure of inhibitors and receptors for docking (see <http://mgltools.scripps.edu>). Two hundred independent docking runs of inhibitors was carried out. After the molecular docking was completed, we clustered analysis of the lowest-energy positions for the inhibitor and then saved the lowest-energy position of the inhibitor in the binding sites of the TE domain.

Statistical analysis

Data were obtained from the mean $\pm$ standard deviation (SD). One-way analysis of variance (ANOVA) and Tukey's post-test were performed by Origin8.5 software (Originlab, MS, USA) to determine the statistical differences among three or more groups. The statistical significance was at $P < 0.05$ level.

Results

ALA reduced the cell viabilities of MDA-MB-231 and MCF-7 cells

To evaluate the cytotoxicity of ALA on human breast cancer cells, MDA-MB-231 and MCF-7 cells were incubated with various concentration of ALA for 24 h. After that, the cell viabilities were detected by MTT assay. ALA treatment for 24 h decreased breast cancer cell viability in a dose-dependent manner, as shown in the Figure 1. The half inhibition concentration (IC_{50}) values of ALA in MDA-MB-231 cells and MCF-7 cells were 45 μ M and 70 μ M, respectively. These results exhibited that ALA could reduce breast cancer cell viability and suppress cell growth.

ALA induced MDA-MB-231 and MCF-7 cells apoptosis

In this study, the apoptotic potential for ALA was assessed in both MDA-MB-231 cells and MCF-7 cells using Annexin V FITC/PI dual staining assay. As shown in the Figure 2A and 2B, the outcomes from the flow cytometric analysis revealed that MDA-MB-231 cells and MCF-7 cells treated with 100 μ M ALA exhibited a significant rise in the percent of Annexin V-FITC positive (Annexin V+) apoptotic cells within both early (from

2.9% to 23.5% and from 5.5 to 16.7%, respectively) and late (from 0.6% to 3.4% and from 2.0 to 13.8%, respectively) apoptotic phases, which involves obvious increase with respect to untreated control cells. We next measured the apoptosis marker proteins PARP and BCL-2. Cells were treated with ALA for 24 h, and then the protein levels were detected by Western blot. As shown in the Figure 2C and 2D, the cleavage PARP increased markedly at 75 μ M in MCF-7 cells and 100 μ M in MDA-MB-231 cells. BCL-2, the anti-apoptotic protein, was down-regulated.

ALA inhibited intercellular FASN in MDA-MB-231 and MCF-7 cells

FASN is concerned to play an important role in breast cancer cell growth. The FASN protein levels were detected by western blot and visualized by immunofluorescence. As shown in the Figure 3A, ALA at a concentration of 80 μ M down-regulated FASN expression significantly in both MDA-MB-231 and MCF-7 cells. To observe the location of FASN at the cytoplasm, the immunofluorescence technology was applied. Results were shown in the Figure 3B, the green fluorescence weakened gradually with the increased of ALA concentration, it indicated that ALA decreased FASN expression with a dose-dependent pattern.

ALA induced endoplasmic reticulum (ER) stress in MDA-MB-231 cells

In order to detect the effect of ALA on ER stress, the expression levels of ER stress markers were detected by Western blot. As shown in Figure 4A, ALA administration for 24 h significantly down-regulated the expression level of FASN, while up-regulated the expression levels of IRE1, ATF6, PERK, and CHOP in MDA-MB-231 cells. To investigate the effect of ALA on mitochondria, mitochondrial membrane potential was measured after ALA treatment. As shown in Figure 4B and 4C, ALA decreased the mitochondrial membrane potential of both MDA-MB-231 cells and MCF-7 cells.

FASN knockdown induced ER stress and decreased cell viability

To identify whether the cell viability and ER stress were related with FASN expression in the breast cancer cells,

we specifically knocked down FASN expression in MDA-MB-231 by siRNA. As shown in Figure 5, the FASN expression was decreased sharply and cell viability was reduced significantly after treatment with siRNA for 24 h. The result demonstrated that decreasing FASN expression could reduce cell viability. Besides, to study whether ER stress will be induced by silencing FASN expression in breast cancer cells, we knocked down the FASN expression in the MDA-MB-231 cells, and then we determined the ER stress level by western blot, the result is shown in Figure 5B.

The cell viability and FASN expression were decreased by ALA rather than PA

In order to confirm whether the effect of ALA on cell viability and FASN expression level is specific, we determined the activity of PA on breast cancer cells as a comparison. After treating with different concentrations of PA or ALA, the cell viabilities of both MDA-MB-231 and MCF-7 cells were detected. The results are shown in Figure 6A, ALA indicated stronger cytotoxicity on breast cancer cells in comparison with PA. Besides, we detected the FASN expression, as shown in Figure 6B, ALA could decrease the FASN expression significantly, while PA did not present the ability to inhibit FAS expression. Those results demonstrate that ALA could inhibit FASN expression and cell viability which could not be affected by PA.

Acetyl-CoA carboxylase inhibition enhanced the cytotoxicity of ALA

For FASN synthesized reaction, acetyl-CoA, malonyl-CoA, and NADPH are applied to produce palmitic acid. Malonyl-CoA comes from acetyl-CoA carboxylase in the cell. For detailed research, TOFA was consumed to inhibit acetyl-CoA carboxylase and to decrease the malonyl-CoA concentration. TOFA is a specific inhibitor of acetyl-CoA carboxylase. As shown in Figure 7, malonyl-CoA concentration was reduced by TOFA, however, ALA showed no significant effect on malonyl-CoA concentration. Besides, the cell viability was decreased a lot after combining with ALA and TOFA.

ALA inhibited the invasion and metastasis, and arrested cell cycle in MDA-MB-231 cells

232 We next detected the invasion of breast cancer cells after treating with ALA for 24 h. Results showed that the
233 cell invasion was inhibited significantly. The cell invasion was almost completely suppressed by 75 μ M ALA.
234 Cell metastasis was measured by transwell assay. Incubation MDA-MB-231 cells with ALA for 24 h, resulted
235 in a dose-dependent inhibition of MDA-MB-231 cells metastasis ability (Figure 8B).

236 To evaluate cell survival, we further checked the effect of ALA on the cell cycle. Incubation MDA-MB-231
237 cells with ALA for 24 h, the result demonstrated that ALA inhibited cell cycle. As the concentration of ALA
238 continues to increase, the proportion of cells in the second peak (G2 phase) decreased. Therefore, ALA could
239 significantly inhibit cell cycle from G2 phase to S phase.

240 **Acetyl-CoA, and malonyl-CoA were not affected after treating with ALA**

241 To further explore whether the substrates of FASN were accumulated after treating ALA for 24 h, intracellular
242 acetyl-CoA, malonyl-CoA, and NADPH concentrations were measured by corresponding kits (Acetyl-CoA kit,
243 Malonyl-CoA kit, and NADPH kit). The result showed that ALA treatment could not increase or decrease the
244 concentrations of both acetyl-CoA and malonyl-CoA.

245 **ALA showed higher affinity with the TE domain than PA**

246 To evaluate the affinity of ALA with TE domain (one of FASN domain). We calculated the binding energy that
247 ALA and PA with TE respectively. Results were shown as the Figure 10A. ALA occupies hydrophobic pocket
248 A and forms a broad hydrophobic interaction with GLU2366, TYR2351, LEU24277, PHE2370, PHE2423,
249 ILE2250, TYR2309 and VAL2344. In addition, the carboxyl group of the ALA forms a salt bridge with the
250 guanidyl groups of ARG2352 and ARG2428. Ser2308-His2481-Asp2338 catalytic triad does not contribute to
251 the binding of ALA. Thus, the primary driving force for the binding of ALA to the TE domain is electrostatic
252 interactions and hydrophobic interactions.

253 The carboxyl group of the PA forms a salt bridge with the guanidyl groups of ARG2352 and ARG2428, and

forms a broad hydrophobic interaction with the residues of pocket A. However, the binding ability of PA is lower than that of ALA in the TE domain.

Discussion

Fatty acids are important sources of energy production and structural components of cells, and also important regulators of gene expression by interacting with various transcription factors. Intake of different types of fatty acids, as well as the total amount of fat, will cause metabolic changes. ALA is an essential omega-3 fatty acid needed for human health. Essential fatty acids are thought to affect several metabolic processes, including regulation of energy supply, enzyme activity and gene expression. Therefore, it is believed that the decrease or imbalance of these lipid levels will have a pathogenic effect on many human diseases. In particular, a large amount of evidence suggests that ALA intake is negatively correlated with coronary and carotid atherosclerotic diseases, and is associated with neuroprotective, antidepressant and antidepressant effects. Many biological functions of ALA have been reported, including cardiovascular protection, neuroprotection, anti-cancer, anti-osteoporosis, anti-inflammatory and antioxidant effects [29]. However, the detailed mechanisms involved remain unclear. In the present study, we proved that ALA could reduce survival rate, induce apoptosis, inhibit cell invasion and metastasis, and block cell cycle in human breast cancer cells.

To gain further insight into the mechanism underlying the anti-cancer effect of ALA, the effect of ALA on intracellular FASN was investigated. Lots of studies proved that ALA could reduce fat accumulation and keep body slim, which provided evidence that ALA could affect fatty acid metabolism [30]. Coincidentally, FASN is a dual target of anti-obesity and anti-cancer [31]. In this study, we found that ALA could down-regulate the expression level of FASN. ALA exhibited higher binding ability to the TE domain of FASN than PA. In order to determine the specificity of ALA on intracellular FASN, we used RNA interference to down-regulate FASN

276 expression in breast cancer cells. The viability of breast cancer cells decreased significantly after the expression
277 of FASN was down-regulated, which was similar to that of ALA treatment. Therefore, ALA may reduce breast
278 cancer cells viability through inhibiting FASN.

279 ER has multiple functions, especially in the synthesis, folding, modification, and transportation. Several
280 enzymes related to lipid metabolism are located in the ER [32, 33]. The ER homeostasis will be destroyed after
281 cells are stimulated, for maintain the balance of microenvironment, apoptosis procedure will be triggered [34].
282 According to our results, the ER stress related proteins were up-regulated, which means ER stress was activated
283 by both ALA treatment and FASN knocking down. The change of ER stress markers is an important mean to
284 reflect mitochondrial stress. We also investigated the effect of ALA on mitochondria. The results showed that
285 ALA induces mitochondrial stress or injury. ER stress response is essential for the survival of cancer cells under
286 some circumstances. However, long-term ER stress and UPR pathway activation can lead to apoptosis. In this
287 study, we found that ALA induced both ER stress and mitochondrial membrane potential reduction. It is strongly
288 suggested that ER stress was involved in ALA induced breast cancer cell death.

289 FASN substrates accumulation should be considered for breast cancer cell apoptosis while FASN was inhibited.
290 NADPH and malonyl-CoA have been reported the apoptosis effect on cancer cells. Excess malonyl-CoA has
291 cytotoxicity for cancer cells. Malonyl-CoA was produced by acetyl-CoA carboxylase and consumed by FASN,
292 malonyl-CoA will be accumulated due to the inhibition of FASN. CPT1 will be inhibited by excess malonyl-
293 CoA, which could trigger apoptosis procedure [35, 36]. We aim to identify whether ALA could induce apoptosis
294 via this way. Acetyl-CoA carboxylase was suppressed by TOFA to decreased the accumulation of malonyl-CoA.
295 According to previous study, cell viability will be remedy by TOFA after inhibiting FASN. One unanticipated
296 finding was that TOFA increased the apoptosis effect which induced by ALA. It is totally different with our
297 prospect. Malonyl-CoA was measured by malonyl-CoA kit, however, the T-test showed that these results were

not statistically significant. A series of experiments show that ALA induced breast cancer cell apoptosis by inhibiting FASN independent malonyl-CoA accumulation. Besides, acetyl-CoA and NADPH also were explored by measuring its concentrations, the result did not present significant difference.

Consistent with the previous research, we found that ALA can also block cell cycle, inhibit the invasion and metastasis of tumor cells [37]. These results prove that ALA can inhibit the development of tumor cells. This also provides a theoretical basis for the development of ALA as an anti-tumor drug or an anti-tumor adjuvant drug. At the same time, it also reminds people that the proper addition of ALA in daily diet is very helpful for maintaining good health. Although we demonstrated the mechanism of ALA induced cancer cell apoptosis, this study remains some limitation. Further animal experiments are urgently needed to determine the feasibility of ALA as an anticancer drug.

Conclusions

In summary, this study suggests that ALA is a novel FASN inhibitor and could induce breast cancer cell apoptosis via inhibiting FASN.

Declarations

Authors' contributions

XM, as the principal investigator, was responsible for the concept and design of the study.

WH, XG, HF, AA, XM, QS, ZL conducted the research and wrote the manuscript.

WH, XG, HF, AA did the whole experiments of the study.

All authors participated in the preparation of, and have approved the final version of the manuscript.

Acknowledgements

320 Not applicable.

321 **Ethics approval and consent to participate**

322 no human subjects.

323 **Consent for publication**

324 Not applicable, no human subjects.

325 **Availability of data and material**

326 All data analyzed and generated during the current study are available from the corresponding author upon
327 reasonable request.

328 **Funding**

329 This work was supported by the Strategic Priority Research Program of the Chinese Academy of Sciences
330 (Grant No. XDA23080601); the Fundamental Research Funds for the Central Universities (Grant No.
331 Y95401AXX2); the Youth Innovation Promotion Association, CAS (Grant No. 2012315); the Beijing
332 Municipal Education Commission (Grant No. KM201910029001).

333 **Competing interests**

334 The authors declare that they have no competing interests.

335 **Disclosure Statement**

336 The authors declare no conflicts of interest.

337

338

References

1. Fan L, Strasser-Weippl K, Li JJ, St Louis J, Finkelstein DM, Yu KD, Chen WQ, Shao ZM, Goss PE. Breast cancer in China. *Lancet Oncol* 2014; 15:e279-89.
2. Peart O. Breast intervention and breast cancer treatment options. *Radiol Technol* 2015; 86:535M-58M; quiz 59-62.
3. Davies K. The Complex Interaction of Matrix Metalloproteinases in the Migration of Cancer Cells through Breast Tissue Stroma. *Int J Breast Cancer* 2014; 2014:839094.
4. Westcott JM, Precht AM, Maine EA, Dang TT, Esparza MA, Sun H, Zhou Y, Xie Y, Pearson GW. An epigenetically distinct breast cancer cell subpopulation promotes collective invasion. *J Clin Invest* 2015; 125:1927-43.
5. Fisusi FA, Akala EO. Drug combinations in breast cancer therapy. *Pharmaceutical Nanotechnology* 2019; 7:3-23.
6. Menendez JA, Lupu R. Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis. *Nat Rev Cancer* 2007; 7:763-77.
7. Menendez JA, Lupu R. Fatty acid synthase (FASN) as a therapeutic target in breast cancer. *Expert Opin Ther Tar* 2017; 21:1001-16.
8. Jones SF, Infante JR. Molecular pathways: fatty acid synthase. *Clin Cancer Res* 2015; 21:5434-8.
9. Mullen GE, Yet L. Progress in the development of fatty acid synthase inhibitors as anticancer targets. *Bioorg Med Chem Lett* 2015; 25:4363-9.
10. Cai Y, Wang J, Zhang L, Wu D, Yu D, Tian X, Liu J, Jiang X, Shen Y, Zhang L, Ren M, Huang P. Expressions of fatty acid synthase and HER2 are correlated with poor prognosis of ovarian cancer. *Medical Oncol* 2015; 32:391.

- 361 11. Papaevangelou E, Almeida GS, Box C, deSouza NM, Chung YL. The effect of FASN inhibition on the
362 growth and metabolism of a cisplatin-resistant ovarian carcinoma model. *Int J Cancer* 2018; 143:992-1002.
- 363 12. Ookhtens M, Kannan R, Lyon I, Baker N. Liver and adipose tissue contributions to newly formed fatty acids
364 in an ascites tumor. *Am J Physiol* 1984; 247:R146-53.
- 365 13. Li P, Tian W, Ma X. Alpha-mangostin inhibits intracellular fatty acid synthase and induces apoptosis in
366 breast cancer cells. *Mol Cancer* 2014; 13:138.
- 367 14. Ventura R, Mordec K, Waszczuk J, Wang Z, Lai J, Fridlib M, Buckley D, Kemble G, Heuer TS. Inhibition
368 of de novo Palmitate Synthesis by Fatty Acid Synthase Induces Apoptosis in Tumor Cells by Remodeling
369 Cell Membranes, Inhibiting Signaling Pathways, and Reprogramming Gene Expression. *EBioMedicine*
370 2015; 2:808-24.
- 371 15. Huang W, Liang Y, Ma X. Alpha-mangostin induces endoplasmic reticulum stress and autophagy which
372 count against fatty acid synthase inhibition mediated apoptosis in human breast cancer cells. *Cancer Cell*
373 *Int* 2019; 19:151.
- 374 16. Puig T, Vázquez-Martín A, Relat J, Pétriz J, Menéndez JA, Porta R, Casals G, Marrero PF, Haro D, Brunet
375 J, Colomer R. Fatty acid metabolism in breast cancer cells: differential inhibitory effects of
376 epigallocatechin gallate (EGCG) and C75. *Breast Cancer Res Tr* 2008; 109:471-9.
- 377 17. Jiang Y, Yin X, Wu L, Qin Q, Xu J. MAPK/P53-mediated FASN expression in bone tumors. *Oncol Lett*
378 2017; 13:4035-8.
- 379 18. Li N, Bu X, Wu P, Wu P, Huang P. The "HER2-PI3K/Akt-FASN Axis" regulated malignant phenotype of
380 colorectal cancer cells. *Lipids* 2012; 47:403-11.
- 381 19. Kim KB, Nam YA, Kim HS, Hayes AW, Lee BM. α -Linolenic acid: nutraceutical, pharmacological and
382 toxicological evaluation. *Food Chem Toxicol* 2014; 70:163-78.

- 383 20. Zhuang P, Shou Q, Wang W, He L, Wang J, Chen J, Zhang Y, Jiao J. Essential fatty acids linoleic acid and
384 α -linolenic acid sex-dependently regulate glucose homeostasis in obesity. *Mol Nutr Food Res* 2018;
385 62:e1800448.
- 386 21. Egert S, Baxheinrich A, Lee-Barkey YH, Tschoepe D, Stehle P, Stratmann B, Wahrburg U. Effects of a
387 hypoenergetic diet rich in α -linolenic acid on fatty acid composition of serum phospholipids in overweight
388 and obese patients with metabolic syndrome. *Nutrition* 2018; 49:74-80.
- 389 22. Zhou X, Wu W, Chen J, Wang X, Wang Y. AMP-activated protein kinase is required for the anti-adipogenic
390 effects of alpha-linolenic acid. *Nutr Metab* 2015; 12:10.
- 391 23. Zhang W, Wang R, Han SF, Bu L, Wang SW, Ma H, Jia GL. Alpha-linolenic acid attenuates high glucose-
392 induced apoptosis in cultured human umbilical vein endothelial cells via PI3K/Akt/eNOS pathway.
393 *Nutrition* 2007; 23:762-70.
- 394 24. Zeng YY, Jiang WD, Liu Y, Wu P, Zhao J, Jiang J, Kuang SY, Tang L, Tang WN, Zhang YA, Zhou XQ, Feng
395 L. Dietary alpha-linolenic acid/linoleic acid ratios modulate intestinal immunity, tight junctions, anti-
396 oxidant status and mRNA levels of NF- κ B p65, MLCK and Nrf2 in juvenile grass carp (*Ctenopharyngodon*
397 *idella*). *Fish Shellfish Immunol* 2016; 51:351-64.
- 398 25. Wang XH, Cui XX, Sun XQ, Wang XH, Li XC, Qi Y, Li W, Han MY, Muhammad I, Zhang XY. High fat
399 diet-induced hepatic 18-carbon fatty acids accumulation up-regulates CYP2A5/CYP2A6 via NF-E2-
400 related factor 2. *Front Pharmacol* 2017; 8:233.
- 401 26. Bavelaar FJ, Beynen AC. Severity of atherosclerosis in parrots in relation to the intake of alpha-linolenic
402 acid. *Avian Dis* 2003; 47:566-77.
- 403 27. Gutierrez-Pajares JL, Ben Hassen C, Oger C, Galano JM, Durand T, Frank PG. Oxidized products of α -
404 linolenic acid negatively regulate cellular survival and motility of breast cancer cells. *Biomolecules* 2019;

405 10:50.

406 28. Mason JK, Klaire S, Kharotia S, Wiggins AK, Thompson LU. α -linolenic acid and docosahexaenoic acid,
407 alone and combined with trastuzumab, reduce HER2-overexpressing breast cancer cell growth but
408 differentially regulate HER2 signaling pathways. *Lipids Health Dis* 2015; 14:91.

409 29. Wiggins AK, Mason JK, Thompson LU. Growth and gene expression differ over time in alpha-linolenic
410 acid treated breast cancer cells. *Exper Cell Res* 2015; 333:147-54.

411 30. Perng W, Villamor E, Mora-Plazas M, Marin C, Baylin A. Alpha-linolenic acid (ALA) is inversely related
412 to development of adiposity in school-age children. *Eur J Clin Nutr* 2015; 69:167-72.

413 31. Wu X, Qin L, Fako V, Zhang JT. Molecular mechanisms of fatty acid synthase (FASN)-mediated resistance
414 to anti-cancer treatments. *Adv Biol Regul* 2014; 54:214-21.

415 32. Schwarz DS, Blower MD. The endoplasmic reticulum: structure, function and response to cellular signaling.
416 *Cell Mol Life Sci* 2016; 73:79-94.

417 33. Suzuki M. Regulation of lipid metabolism via a connection between the endoplasmic reticulum and lipid
418 droplets. *Anat Sci Int* 2017; 92:50-4.

419 34. Fernández A, Ordóñez R, Reiter RJ, González-Gallego J, Mauriz JL. Melatonin and endoplasmic reticulum
420 stress: relation to autophagy and apoptosis. *J Pineal Res* 2015; 59:292-307.

421 35. Zhou W, Tu Y, Simpson PJ, Kuhajda FP. Malonyl-CoA decarboxylase inhibition is selectively cytotoxic to
422 human breast cancer cells. *Oncogene* 2009; 28:2979-87.

423 36. Thupari JN, Pinn ML, Kuhajda FP. Fatty acid synthase inhibition in human breast cancer cells leads to
424 malonyl-CoA-induced inhibition of fatty acid oxidation and cytotoxicity. *Biochem Biophys Res Commun*
425 2001; 285:217-23.

426 37. du Toit PJ, van Aswegen CH, du Plessis DJ. The effect of essential fatty acids on growth and urokinase-type

427 plasminogen activator production in human prostate DU-145 cells. Prostaglandins Leukot Essent Fatty
428 Acids 1996; 55:173-7.
429

Figure Legends

Figure 1. Effect of ALA on breast cancer cell viabilities.

MDA-MB-231 cells (A) and MCF-7 cells (B) were treated by different concentrations of ALA (0, 10, 30, 50, 60, 70, 80, 90, and 100 μ M) for 24 h, cell viability was measured by MTT assay. Data represented the mean $\pm$ SD of three independent experiments. MBA-MB-231 cells.

Figure 2. Apoptotic effect of ALA on MCF-7 and MDA-MB-231 cells.

(A) MDA-MB-231 cells and MCF-7 cells were treated with various concentrations of ALA for 24 h, then incubated and stained with annexin V/PI, analyzed by flow cytometry. (B) Apoptosis in MDA-MB-231 cells and MCF-7 cells was assessed by PARP cleavage and BCL-2 expression. GAPDH was chosen as a loading control.

Figure 3. ALA down-regulated intercellular FASN expression in breast cancer cells.

(A) The expression levels of FASN after ALA treatment for 24 h in MDA-MB-231 and MCF-7 cells were detected. FASN was stained by FASN antibody with FITC (green fluorescence), nucleus was stained by DAPI (blue fluorescence). The image was analyzed by fluorescence microscope. (B) The expression levels of FASN were analyzed by western blot after ALA treatment.

Figure 4. The effects of ALA on ER stress in MDA-MB-231 cells.

(A) At 24 h after ALA (100 μ M) treatment, the relative expression levels of FASN, ATF6, IRE1, PERK, and CHOP were analyzed by western blot. (B) MDA-MB-231 cells and (C) MCF-7 cells were treated with ALA (0, 25, 50, 75, and 100 μ M) analyzed by measuring the fluorescence shift of JC-1 with a flow cytometer to assess the mitochondrial dysfunction.

Figure 5. Down-regulating FASN expression reduced cell viability and induced ER stress in MDA-MB-231 cells.

(A) MDA-MB-231 cells were transfected with FASN targeted siRNA for 24 h. The survival rate of cells was

measured by MTT assay. (B) The relative expression levels of ATF6, IRE1, PERK, and CHOP in MDA-MB-231 cells were analyzed by Western blot. The density of proteins was quantified by ImageJ software. * $p < 0.05$.

Figure 6. The effects of ALA, PA on cell viability and FASN expression level in breast cancer cells.

Various concentration of ALA or PA (0, 25, 50, 70, and 100 μM) treated MDA-MB-231 cells (A) and MCF-7 cells (B) for 24 h, cell viabilities were measured by MTT assay. * $p < 0.05$, ** $p < 0.01$. (C) and (D) FASN expression was analyzed by western blot after treating with ALA or PA for 24 h.

Figure 7. The effects of ALA and TOFA on cell viability in breast cancer cells.

MDA-MB-231 cells and MCF-7 cells were treated with ALA alone or ALA accompany with TOFA for 24 h,

cell viabilities were measured by MTT assay. * $p < 0.05$, ** $p < 0.01$.

Figure 8. ALA inhibited the invasion and arrested the cell cycle of MDA-MB-231 cells.

MDA-MB-231 cells were treated with ALA (0, 25, 50, 75, and 100 μ M) for 24 h. (A) Wound healing experiment.

(B) Transwell assay. (C) Cell cycles were analyzed.

Figure 9. Effects of ALA on the FASN substrate.

Three substrates involved in the FASN reaction. MDA-MB-231 and MCF-7 cells were treated ALA (0, 25, 50, 75, and 100 μM) for 24 h, corresponding kits (Acetyl-CoA kit, Malonyl-CoA kit, and NADPH kit) were used to measure acetyl-CoA, malonyl-CoA, and NADPH, respectively. * $p < 0.05$, ** $p < 0.01$.

Figure 10. Molecular docking for ALA and PA with TE domain of FASN.

(A) The distribution of binding energy for ALA with TE domain which calculated by Autodock Vina, the red column was analyzed by Pymol. (B) The binding model of ALA with TE domain. (C) The distribution of binding energy for PA with TE domain which calculated by Autodock Vina, the red column was analyzed by Pymol as shown in the figure D. (D) The binding model of PA with TE domain.

A	B
C	D

491

Figures

Figure 1

Effect of ALA on breast cancer cell viabilities. MDA-MB-231 cells (A) and MCF-7 cells (B) were treated by different concentrations of ALA (0, 10, 30, 50, 60, 70, 80, 90, and 100 μ M) for 24 h, cell viability was measured by MTT assay. Data represented the mean $\pm$ SD of three independent experiments. MBA-MB-231 cells.

Figure 2

Apoptotic effect of ALA on MCF-7 and MDA-MB-231 cells. (A) MDA-MB-231 cells and MCF-7 cells were treated with various concentrations of ALA for 24 h, then incubated and stained with annexin V/PI, analyzed by flow cytometry. (B) Apoptosis in MDA-MB-231 cells and MCF-7 cells was assessed by PARP cleavage and BCL-2 expression. GAPDH was chosen as a loading control.

Figure 3

ALA down-regulated intercellular FASN expression in breast cancer cells. (A) The expression levels of FASN after ALA treatment for 24 h in MDA-MB-231 and MCF-7 cells were detected. FASN was stained by FASN antibody with FITC (green fluorescence), nucleus was stained by DAPI (blue fluorescence). The image was analyzed by fluorescence microscope. (B) The expression levels of FASN were analyzed by western blot after ALA treatment.

Figure 4

The effects of ALA on ER stress in MDA-MB-231 cells. (A) At 24 h after ALA (100 μ M) treatment, the relative expression levels of FASN, ATF6, IRE1, PERK, and CHOP were analyzed by western blot. (B) MDA-

MB-231 cells and (C) MCF-7 cells were treated with ALA (0, 25, 50, 75, and 100 μ M) analyzed by measuring the fluorescence shift of JC-1 with a flow cytometer to assess the mitochondrial dysfunction.

Figure 5

Down-regulating FASN expression reduced cell viability and induced ER stress in MDA-MB-457 231 cells. (A) MDA-MB-231 cells were transfected with FASN targeted siRNA for 24 h. The survival rate of cells was measured by MTT assay. (B) The relative expression levels of ATF6, IRE1, PERK, and CHOP in MDA-MB-231 cells were analyzed by Western blot. The density of proteins was quantified by ImageJ software. * $p < 0.05$.

Figure 6

The effects of ALA, PA on cell viability and FASN expression level in breast cancer cells. Various concentration of ALA or PA (0, 25, 50, 70, and 100 μ M) treated MDA-MB-231 cells (A) and MCF-7 cells (B) for 24 h, cell viabilities were measured by MTT assay. * $p < 0.05$, ** $p < 0.01$. (C) and (D) FASN expression was analyzed by western blot after treating with ALA or PA for 24 h.

Figure 7

The effects of ALA and TOFA on cell viability in breast cancer cells. MDA-MB-231 cells and MCF-7 cells were treated with ALA alone or ALA accompany with TOFA for 24 h, cell viabilities were measured by MTT assay. * $p < 0.05$, ** $p < 0.01$.

Figure 8

ALA inhibited the invasion and arrested the cell cycle of MDA-MB-231 cells. MDA-MB-231 cells were treated with ALA (0, 25, 50, 75, and 100 μM) for 24 h. (A) Wound healing experiment. (B) Transwell assay. (C) Cell cycles were analyzed.

Figure 9

Effects of ALA on the FASN substrate. Three substrates involved in the FASN reaction. MDA-MB-231 and MCF-7 cells were treated ALA (0, 25, 50, 75, and 100 μM) for 24 h, corresponding kits (Acetyl-CoA kit, Malonyl-CoA kit, and NADPH kit) were used to measure acetyl-CoA, malonyl-CoA, and NADPH, respectively. *p < 0.05, **p < 0.01.

A	B
C	D

Figure 10

Molecular docking for ALA and PA with TE domain of FASN. (A) The distribution of binding energy for ALA with TE domain which calculated by Autodock Vina, the red column was analyzed by Pymol. (B) The binding model of ALA with TE domain. (C) The distribution of binding energy for PA with TE domain which calculated by Autodock Vina, the red column was analyzed by Pymol as shown in the figure D. (D) The binding model of PA with TE domain.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplementaryoriginaldata.pdf](#)