

ORIGINAL RESEARCH ARTICLE

Phospholipid distribution of endogenous geranylgeranoic acid and its zaragozic acid A-induced shift toward cardiolipin-enriched fractions

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Abstract

Introduction: Geranylgeranoic acid (GGA) is an endogenous lipid mediator implicated in hepatocellular carcinogenesis, but its phospholipid-associated intracellular distribution remains poorly defined.

Objectives: To characterize the lipid-fraction distribution of endogenous GGA in HuH-7 hepatoma cells and determine whether enhanced non-sterol isoprenoid flux alters its recovery from phospholipid-associated fractions.

Methods: HuH-7 cells were analyzed under basal conditions and after treatment with squalenstatin-1/zaragozic acid A (ZAA), a squalene synthase inhibitor. Cellular lipids were separated by thin-layer chromatography (TLC), followed by alkaline hydrolysis and GGA quantification using liquid chromatography-tandem mass spectrometry. Polar lipids were further separated to assess recovery from phosphatidylcholine (PC), phosphatidylethanolamine (PE), and solvent-front/cardiolipin (CL)-enriched fractions. Supplementary CL-resolving TLC was also performed.

Results: Under basal conditions, GGA was recovered mainly from polar lipids, predominantly in PC and, to a lesser extent, PE fractions, whereas the solvent-front/CL-enriched fraction contained only minor amounts. ZAA increased GGA recovery 6.0-fold from the free fatty acid fraction and 2.2-fold from the polar lipid fraction. Within phospholipid-containing fractions, ZAA increased recovery 18.2-fold from the solvent-front/CL-enriched fraction, while PC remained relatively unchanged and PE decreased modestly. Supplementary CL-resolving analysis supported recovery from a CL-associated fraction.

Conclusion: GGA was recovered after alkaline hydrolysis of TLC fractions; however, this does not establish covalent esterification or identify the relevant intact molecular species. Increased intracellular GGA availability was accompanied by expansion of a CL-associated recoverable pool, supporting future investigation of its relationship to mitochondrial lipid remodeling and hepatoma-cell stress responses.

Keywords: Geranylgeranoic acid; Cardiolipin; Phosphatidylcholine; Phosphatidylethanolamine; Zaragozic acid A; LC-MS/MS

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1. Introduction

Geranylgeranoic acid (GGA) is a naturally occurring acyclic diterpenoid endogenously synthesized via the mevalonate (MVA) pathway in mammalian cells.¹⁻⁶ While GGA was initially studied as a differentiation-inducing agent acting through retinoic acid receptors (RAR/RXR),⁷ GGA and related acyclic retinoids have more recently been implicated in hepatic tumor suppression.^{5,8,9} *In vivo* studies suggest that decreased hepatic GGA levels are associated with the onset of spontaneous hepatocellular carcinoma in aged rodents,^{5,10,11} whereas dietary supplementation of GGA reduces tumor incidence in carcinogen-induced and spontaneous hepatoma models.^{5,10,11} In parallel, exogenous GGA induces apoptosis- or pyroptosis-like cell death selectively in human hepatoma-derived cell lines,¹²⁻¹⁵ but not in normal hepatocytes.³ These findings support the potential tumor-suppressive function of GGA; however, the mechanistic link between endogenous GGA biosynthesis, intracellular handling, and hepatoma-cell stress responses remains incompletely understood.

Recent studies have broadened the biological context of GGA from a synthetic acyclic retinoid to a lipid mediator with dual endogenous and dietary origins.¹⁶ GGA has been detected in plant-derived foods, including turmeric and selected nuts and legumes, suggesting that circulating and hepatic GGA levels may be influenced not only by endogenous biosynthesis but also by habitual dietary intake.² At the same time, mammalian GGA biosynthesis has been linked to the metabolism of prenols and prenoic acids, a group of acyclic isoprenoid metabolites that are increasingly recognized as underexplored regulators of metabolism, disease prevention, and aging.^{4,5} This dual origin is particularly relevant to hepatic tumor suppression, because age-related changes in GGA availability may depend on both dietary supply and endogenous isoprenoid metabolism.

Endogenous GGA is biosynthesized from geranylgeranyl pyrophosphate (GGPP), which is derived from farnesyl pyrophosphate (FPP), a downstream metabolite of the MVA pathway.¹ Under physiological conditions, FPP is primarily utilized for cholesterol biosynthesis,¹⁷ whereas GGPP is mainly used for protein prenylation and also serves as a precursor for dolichols and ubiquinone.¹⁸⁻²⁰ A key branching point in the MVA pathway is catalyzed by squalene synthase (SQS), which converts FPP into squalene and thereby commits the metabolic flux toward sterol biosynthesis.^{17,18} As a result, only a limited fraction of isoprenoid intermediates may be available for GGA biosynthesis under basal conditions. Pharmacological inhibition of SQS has therefore been used to increase non-sterol isoprenoid flux and enhance

endogenous GGA production.¹ Squalostatins, also known as zaragozic acid A (ZAA), diverts FPP away from sterol biosynthesis toward non-sterol branches of the MVA pathway, resulting in increased levels of non-sterol isoprenoids, including GGA.^{1,17,21} Thus, SQS inhibition provides a useful experimental approach for examining how increased endogenous GGA availability affects its intracellular lipid distribution.

The enzymatic regulation of endogenous GGA biosynthesis has also attracted attention in the context of aging and hepatocarcinogenesis. Previous studies have identified monoamine oxidase B (MAOB) as a major enzyme involved in the oxidative metabolism of geranylgeraniol to GGA, whereas cytochrome P450 3A4 (CYP3A4) may provide a compensatory pathway under MAOB-deficient conditions.^{4,5,13} Because hepatic MAOB expression and activity may decline with aging, reduced MAOB-dependent GGA synthesis has been proposed as a potential contributor to age-related reduction of hepatic GGA levels and increased susceptibility to spontaneous hepatocarcinogenesis.^{4,5,13,22} More recently, MAOB has been discussed as a regulator of endogenous GGA production in hepatocytes, in addition to its established role as a mitochondrial redox enzyme.^{4,5,13} These observations further emphasize the need to understand how newly synthesized GGA is handled within intracellular lipid pools.

Previous studies have generally treated reduced endogenous GGA during tumorigenesis^{5,13,16} and exogenous GGA-induced tumor-selective cell death^{3,12} as related but distinct phenomena. One possible connection between these observations is that endogenously produced GGA may behave, at least in part, as a lipid mediator whose activity is regulated by intracellular lipid pools. Classical lipid mediators, such as arachidonic acid-derived prostaglandins and leukotrienes, are stored in membrane phospholipids and released by enzymatic hydrolysis in response to cellular stimuli.²³⁻²⁶ By analogy, GGA may also be stored or handled in hydrolysable phospholipid-associated pools, from which it could be mobilized under metabolic or stress conditions. Although GGA is structurally distinct from polyunsaturated fatty acid-derived lipid mediators, its reported biological actions beyond classical nuclear receptor activation, including modulation of retinoid signaling and hepatoma-cell stress responses,^{7,13,14} support the need to define its intracellular lipid distribution.

Such compartmentalization within specific phospholipid classes may provide spatial and temporal regulation of GGA activity. In particular, changes in the recovery of GGA from mitochondria-associated lipid fractions may be relevant to

hepatoma-cell stress responses, because cardiolipin (CL) is a mitochondria-associated phospholipid involved in mitochondrial membrane organization, respiratory chain function, cristae integrity, and cell-death regulation.²⁷⁻²⁹ CL remodeling is also closely linked to mitochondrial stress responses, and alterations in CL composition can influence mitochondrial vulnerability under pathological conditions.²⁹⁻³³ Therefore, if endogenous GGA is recovered from CL-enriched or CL-associated lipid fractions, such a distribution may provide a biochemical connection between isoprenoid metabolism and mitochondrial lipid remodeling in hepatoma cells.

However, it remains unclear whether endogenous GGA is primarily associated with common membrane phospholipids under basal conditions, or whether increased GGA production alters its phospholipid-fraction distribution. In particular, it is unknown whether enhancement of non-sterol isoprenoid flux causes a broad increase in free GGA alone, or whether a portion of GGA becomes recoverable from selected hydrolysable phospholipid-associated pools. In this study, the lipid-class distribution of endogenous GGA in HuH-7 hepatoma cells by thin-layer chromatography (TLC), alkaline saponification, and LC-MS/MS was examined. Whether enhancement of non-sterol isoprenoid flux by ZAA alters the recovery of GGA from major phospholipid fractions, including phosphatidylcholine (PC), phosphatidylethanolamine (PE), and solvent-front/CL-enriched fractions, was further investigated.

2. Material and methods

2.1. Chemicals and reagents

Geranylgeranoic acid (GGA) was prepared by Kuraray Co. (Okayama, Japan) and Kowa Pharmaceutical Co. Ltd. (Tokyo, Japan). Zaragozic acid A (ZAA; squalstatin-1) was obtained from Merck (Darmstadt, Germany). Authentic lipid standards used for TLC band identification, including phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), cardiolipin (CL), free fatty acid (FFA), triacylglycerol (TG), diacylglycerol (DG), and cholesteryl ester (ChE), were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture reagents were obtained as follows: High-glucose Dulbecco's Modified Eagle Medium (DMEM) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Fetal bovine serum (FBS) was purchased from HyClone, Thermo Fisher Scientific (Tokyo, Japan). Dulbecco's phosphate-buffered saline without calcium chloride and magnesium chloride [PBS(-)] and other cell culture reagents were purchased from Sigma-Aldrich.

Organic solvents and TLC-related reagents were obtained from commercial sources. Chloroform, n-hexane, and methanol were purchased from Kanto Chemical Co. (Tokyo, Japan). Diethyl ether, acetic acid, potassium hydroxide (KOH), hydrochloric acid (HCl), and iodine were purchased from Wako Pure Chemical Industries. Acetonitrile of LC/MS grade, ethanol, and triethylamine were purchased from Sigma-Aldrich. Silica gel 60 TLC plates were obtained from Merck. All other chemicals were of analytical or reagent grade.

2.2. Cell culture

The human hepatoma cell line HuH-7 (Cell Bank, RIKEN, Wako, Saitama, Japan) was cultured in high-glucose DMEM supplemented with 5% fetal bovine serum (FBS). Cells were maintained at 37 °C in a humidified incubator with 5% CO₂. For experiments, HuH-7 cells were seeded at a density of 5×10^5 cells per 100-mm dish and allowed to adhere for 24 h in DMEM containing 5% FBS. Cells were used for experiments when they reached approximately 80–90% confluence.

2.3. ZAA treatment

One day prior to treatment, the culture medium was replaced with serum-free DMEM. Cells were then exposed to 2.5 μM ZAA for 48 h. ZAA was dissolved in ethanol and added to cultures at a final solvent concentration of 0.1% (v/v). Control cells received 0.1% ethanol vehicle without ZAA. Previous work confirmed that 2.5 μM ZAA does not induce significant cytotoxicity in HuH-7 cells within 48 h.¹ Following treatment, cells were collected using a plastic cell lifter (Nunc, Roskilde, Denmark), pelleted, and weighed to determine cell wet weight. GGA content was normalized to cell wet weight and expressed as ng/g cell wet weight.

2.4. Lipid extraction

Total lipids were extracted from cultured cells using a chloroform/methanol (2:1, v/v) mixture according to the Folch method,³⁴ with minor modifications. The lipid extracts were dried under a stream of nitrogen and re-dissolved in a small volume of chloroform for subsequent TLC separation.

2.5. TLC(1): neutral and polar lipid separation

The total lipid extract was applied onto silica gel 60 TLC plates (Merck) and developed with a solvent system consisting of n-hexane: diethyl ether: acetic acid (80:20:1, v/v/v). This system was used to separate free fatty acids (FFA), triacylglycerol (TG), cholesteryl ester (ChE), diacylglycerol (DG), and the origin fraction containing mainly polar lipids, including phospholipids. Lipid bands were visualized by brief exposure to iodine vapor and

identified by comparison with authentic lipid standards. The corresponding bands were scraped from the TLC plates and subjected to alkaline hydrolysis followed by LC-MS/MS analysis.

2.6. TLC(2): phospholipid-class separation

Polar lipids recovered from the origin fraction of TLC(1) were extracted with chloroform: methanol (2:1, v/v) and further separated by TLC using a solvent system consisting of n-hexane: methanol: water: triethylamine (90:15:10:1, v/v/v/v). This solvent system was used as the primary phospholipid-class separation system because it allowed simultaneous fractionation of major phospholipid-containing fractions, including the solvent-front fraction, PE, PI, PS, PC, and lysophospholipids. The solvent-front fraction was operationally designated as a CL-enriched fraction based on the migration of an authentic CL standard under the same TLC condition. This designation indicates co-migration with the CL standard under the present TLC conditions and does not establish chemical purity of the scraped fraction or exclude co-migrating lipid species. After development, lipid bands were visualized by brief exposure to iodine vapor, scraped from the TLC plate according to the migration of authentic lipid standards, and subjected to alkaline hydrolysis followed by LC-MS/MS analysis.

For supplementary confirmation of CL-associated GGA recovery, polar lipid extracts were also analyzed using a CL-resolving TLC solvent system consisting of chloroform, methanol, acetic acid, and water (85:15:10:3.5, v/v/v/v). This solvent system was used to separate CL, PE, monolysocardiolipin (MLCL), and lysophosphatidylethanolamine (LPE), based on the migration of authentic lipid standards. The corresponding bands were scraped from the TLC plate and subjected to alkaline hydrolysis followed by LC-MS/MS analysis. Because this CL-resolving solvent system did not resolve PC, these data were used as supplementary evidence supporting GGA recovery from a fraction co-migrating with authentic CL, rather than as definitive identification of GGA-containing CL molecular species.

2.7. Alkaline hydrolysis of TLC-scraped lipid fractions

TLC-scraped lipid fractions were extracted and evaporated to dryness under nitrogen. The dried residues were re-dissolved in 100 μ L ethanol. Alkaline hydrolysis was performed by adding 5 M KOH and heating at 56 $^{\circ}$ C for 1 h to recover GGA released under the present alkaline hydrolysis conditions. The reaction mixture was cooled on ice, neutralized with acetic acid and HCl, and extracted with n-hexane. The organic phase was evaporated under

nitrogen, and the residue was re-dissolved in 100 μ L ethanol for LC-MS/MS analysis.

2.8. LC/MS/MS analysis

Quantification of GGA in each TLC fraction was performed using a Waters Acquity UPLC system coupled to a tandem quadrupole mass spectrometer (Waters, Milford, MA, USA). The mass spectrometer was operated in electrospray ionization negative mode, and GGA was detected using multiple reaction monitoring (MRM), as described in previous studies.^{1,2} Chromatographic separation, mobile phase composition, gradient program, MRM conditions, and instrumental parameters were identical to those reported previously.^{1,2} GGA in TLC-scraped fractions was identified based on comparison with an authentic GGA standard, including chromatographic retention time and MRM detection profile. Quantification was performed using an external calibration curve generated with authentic GGA standards analyzed under the same LC-MS/MS conditions. No fraction-specific internal standard, spike-recovery correction, or formal mass-balance correction was applied. Because a stable isotope-labeled GGA internal standard was not used, no correction was made for fraction-specific matrix effects, ion suppression, silica-dependent recovery, hydrolysis efficiency, or extraction losses. The amount of GGA detected in each fraction was normalized to cell wet weight and expressed as ng/g cell wet weight.

2.9. Statistical analysis

All experiments were performed in three independent replicates. Data are expressed as mean $\pm$ SD. Comparisons between vehicle-treated and ZAA-treated cells were performed using Welch's *t*-test with GraphPad Prism 11. Welch's *t*-test was selected because it does not assume equal variances. Owing to the limited sample size, normality and variance homogeneity were not formally assessed. No adjustment for multiple comparisons or formal effect-size confidence intervals was applied. Therefore, *p*-values < 0.05 were interpreted as exploratory evidence of differences between treatment conditions.

3. Results

3.1. Endogenous GGA was mainly recovered from the polar lipid fraction under basal conditions

To examine the lipid-class distribution of endogenous GGA in HuH-7 hepatoma cells, total lipid extracts from vehicle-treated control cells were first separated into neutral lipid fractions and the origin fraction by TLC. LC-MS/MS analysis after alkaline hydrolysis showed that GGA was mainly recovered from the origin fraction, which

contained polar lipids including phospholipids (Table 1). The GGA concentration in the origin fraction was 74.1 ± 7.9 ng/g cell wet weight. Lower levels were detected in the free fatty acid (FFA) fraction (14.1 ± 2.8 ng/g cell wet weight) and triacylglycerol (TG) fraction (2.6 ± 0.6 ng/g cell wet weight), whereas GGA was not detected in the diacylglycerol (DG), sterol, or solvent-front fractions.

Because free GGA migrates with the FFA fraction under this neutral lipid TLC condition, the predominant recovery of GGA from the origin fraction suggested that a substantial portion of endogenous GGA was associated with polar lipid fractions rather than being present solely as free GGA. Therefore, the origin fraction was further subjected to phospholipid-class separation by a second TLC system.

3.2. GGA was predominantly recovered from PC and PE fractions under basal conditions

To determine the phospholipid-fraction distribution of endogenous GGA, polar lipids recovered from the origin fraction of TLC(1) were further separated by TLC(2). The TLC(2) system separated major phospholipid-containing fractions, including the cardiolipin-enriched solvent-front fraction, lysophospholipid-containing fraction, phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE), and the origin fraction. The cardiolipin-enriched solvent-front fraction is hereafter referred to as the CL-enriched fraction.

In vehicle-treated control cells, GGA was predominantly recovered from the PC fraction, with a concentration of 38.5 ± 4.2 ng/g cell wet weight (Table 1). PE also contained detectable GGA at 11.2 ± 3.6 ng/g cell wet weight. In contrast, the CL-enriched fraction contained a smaller amount of GGA, 4.8 ± 1.2 ng/g cell wet weight. GGA was not detected in the origin, lysophospholipid, PS, or PI fractions under the present analytical conditions. These results indicate that, under the present TLC fractionation conditions, GGA recovery after alkaline hydrolysis was greatest in the PC and PE fractions, whereas recovery from the solvent-front/CL-enriched fraction was limited.

3.3. ZAA treatment increased GGA recovery in both the polar lipid and FFA fractions

To examine whether enhanced non-sterol isoprenoid flux alters the lipid-class distribution of GGA, HuH-7 cells were treated with $2.5 \mu\text{M}$ ZAA for 48 h. Total lipid extracts were separated by TLC(1), and GGA was quantified after alkaline hydrolysis. ZAA treatment increased GGA recovery in the origin fraction from 74.1 ± 7.9 to 160.2 ± 26.8 ng/g cell wet weight (Table 2), corresponding to an

approximately 2.2-fold increase compared with vehicle-treated control cells. GGA recovery in the FFA fraction also increased markedly, from 14.1 ± 2.8 to 84.6 ± 13.5 ng/g cell wet weight, representing an approximately 6.0-fold increase. In contrast, the TG fraction showed only a small increase, from 2.6 ± 0.6 to 3.5 ± 0.9 ng/g cell wet weight, and GGA remained undetectable in the DG, sterol, and solvent-front fractions.

Table 1. Distribution of endogenous GGA in lipid fractions of vehicle-treated HuH-7 cells

TLC system	Fraction	GGA (ng/g cell wet weight)
TLC(1)	Solvent front	N.D.
	ChE	N.D.
	TG	2.6 ± 0.6
	FFA	14.1 ± 2.8
	DG	N.D.
	Sterol	N.D.
	Origin (polar lipids)	74.1 ± 7.9
	Solvent front / CL-enriched	4.8 ± 1.2
	PE	11.2 ± 3.6
	PC	38.5 ± 4.2
TLC(2)	LPL	N.D.
	PS	N.D.
	PI	N.D.
	Origin	N.D.

Notes: Values are expressed as mean $\pm$ SD from three independent experiments. GGA was quantified by LC-MS/MS after alkaline hydrolysis of TLC-scraped fractions.

Abbreviations: ChE: Cholesteryl ester; CL: Cardiolipin; DG: Diacylglycerol; FFA: Free fatty acid; GGA: Geranylgeranoic acid; LPL: Lysophospholipids; N.D.: Not detected; PC: Phosphatidylcholine; PE: Phosphatidylethanolamine; PI: Phosphatidylinositol; PS: Phosphatidylserine; Sterol: Free sterol fraction; TG: Triacylglycerol.

These findings indicate that ZAA-induced enhancement of non-sterol isoprenoid flux increased cellular GGA recovery in both the polar lipid fraction and the FFA fraction. The increase in the FFA fraction is consistent with an increase in free or FFA-migrating GGA, whereas the prominent increase in the origin fraction suggests that a substantial portion of ZAA-induced GGA was also recovered from polar lipid-associated pools. Therefore, the origin fraction was further analyzed by phospholipid-class

TLC to determine whether ZAA altered the distribution of GGA among specific phospholipid-containing fractions.

3.4. ZAA treatment markedly increased GGA recovery from the CL-enriched fraction

The origin fraction from ZAA-treated cells was further separated by TLC(2) to determine whether ZAA altered the phospholipid-fraction distribution of GGA. ZAA treatment caused a marked increase in GGA recovery from the solvent-front/CL-enriched fraction, from 4.8 ± 1.2 to 87.4 ± 12.4 ng/g cell wet weight (Table 2), corresponding to an approximately 18-fold increase compared with vehicle-treated control cells. In contrast, GGA recovery from the PC fraction remained largely unchanged, increasing only slightly from 38.5 ± 4.2 to 41.9 ± 5.1 ng/g cell wet weight. GGA recovery from the PE fraction showed a modest decrease, from 11.2 ± 3.6 to 9.8 ± 2.9 ng/g cell wet weight. GGA was not detected in the origin, lysophospholipid, PS, or PI fractions under the present analytical conditions.

Thus, ZAA treatment did not cause a uniform increase in GGA recovery across the analyzed phospholipid-containing fractions. Rather, the largest increase in GGA recovery was observed in the solvent-front/CL-enriched fraction, whereas PC-associated recovery was maintained and PE-associated recovery was only slightly reduced. Representative LC-MS/MS chromatograms from ZAA-treated HuH-7 cells confirmed GGA-related peaks in the solvent-front/CL-enriched, PE, and PC fractions after alkaline hydrolysis (Figure 1). These chromatographic profiles were consistent with the quantitative data showing prominent GGA recovery from the solvent-front/CL-enriched fraction and detectable recovery from PC and PE fractions.

Polar lipid extracts were also analyzed using a CL-resolving TLC solvent system. In this supplementary analysis, GGA was recovered from the CL fraction at 92.6 ± 17.5 ng/g cell wet weight, a level comparable to that observed in the solvent-front/CL-enriched fraction obtained using the primary phospholipid TLC system (87.4 ± 12.4 ng/g cell wet weight). PE-associated GGA recovery in the CL-resolving system (11.7 ± 4.6 ng/g cell wet weight) was also similar to that observed in the primary TLC(2) system (9.8 ± 2.9 ng/g cell wet weight), whereas MLCL and LPE showed no detectable GGA (Table A1, see Appendix). Because this CL-resolving system did not quantify PC, these data were used as supportive evidence and are presented separately from the main phospholipid-fraction distribution analysis. These changes were also visualized in Figure 2. In TLC(1), ZAA treatment significantly increased GGA recovery from the origin/polar lipid fraction and the FFA fraction, whereas the TG

fraction did not show a significant increase. In TLC(2), ZAA treatment significantly increased GGA recovery from the solvent-front/CL-enriched fraction, whereas the PC and PE fractions did not show significant increases. These results indicate that ZAA-induced GGA accumulation was not uniformly distributed across lipid fractions but was characterized by significant increases in the FFA and solvent-front/CL-enriched fractions.

Table 2. Distribution of GGA in lipid fractions of 2.5 μ M ZAA-treated HuH-7 cells

TLC system	Fraction	GGA (ng/g cell wet weight)
TLC(1)	Solvent front	N.D.
	ChE	N.D.
	TG	3.5 ± 0.9
	FFA	84.6 ± 13.5
	DG	N.D.
	Sterol	N.D.
	Origin (polar lipids)	160.2 ± 26.8
	Solvent front / CL-enriched	87.4 ± 12.4
	PE	9.8 ± 2.9
	PC	41.9 ± 5.1
TLC(2)	LPL	N.D.
	PS	N.D.
	PI	N.D.
	Origin	N.D.

Notes: HuH-7 cells were treated with 2.5 μ M ZAA for 48 h. Values are expressed as mean $\pm$ SD from three independent experiments. GGA was quantified by LC-MS/MS after alkaline hydrolysis of TLC-scraped fractions.

Abbreviations: ChE: Cholesteryl ester; CL: Cardiolipin; DG: Diacylglycerol; FFA: Free fatty acid; GGA: Geranylgeranoic acid; LPL: Lysophospholipids; N.D.: Not detected; PC: Phosphatidylcholine; PE: Phosphatidylethanolamine; PI: Phosphatidylinositol; PS: Phosphatidylserine; Sterol: Free sterol fraction; TG: Triacylglycerol; ZAA: Zaragozic acid A.

4. Discussion

This study provides an initial characterization of the lipid-fraction distribution of endogenous GGA in HuH-7 hepatoma cells. Under basal conditions, GGA was mainly recovered from the polar lipid fraction, and subsequent phospholipid-class separation showed predominant recovery from PC and, to a lesser extent, PE fractions. The

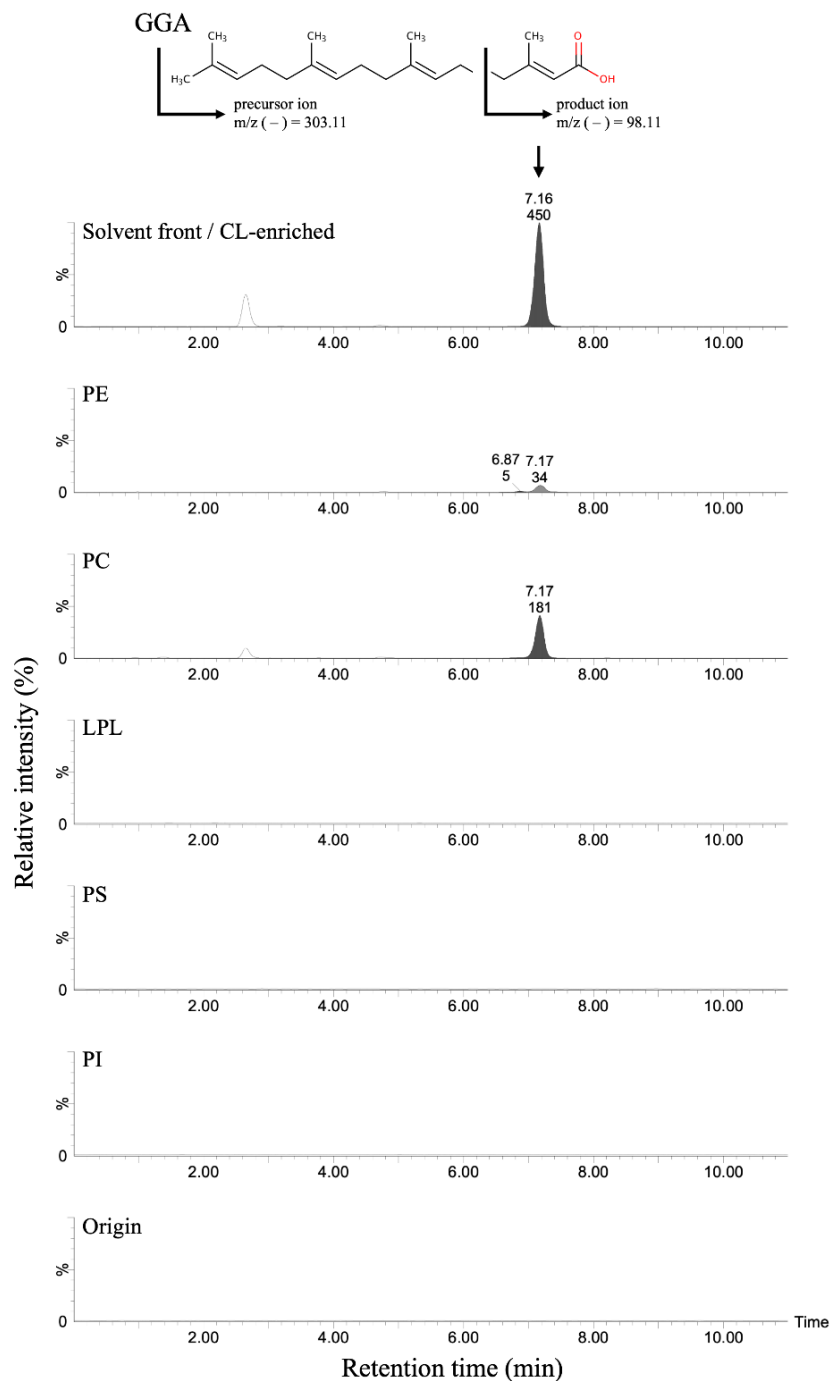


Figure 1. Representative LC-MS/MS chromatograms of GGA recovered from phospholipid-containing fractions of ZAA-treated HuH-7 cells. HuH-7 cells were treated with 2.5 μ M ZAA for 48 h, and polar lipid fractions were separated by TLC(2). Each TLC-scraped fraction was subjected to alkaline hydrolysis, followed by LC-MS/MS analysis of GGA. GGA-related peaks were detected at approximately 7.16–7.17 min in the solvent-front/CL-enriched, PC, and PE fractions, whereas little or no signal was observed in LPL, PS, PI, and origin fractions. The chromatograms are representative of three independent experiments.

Abbreviations: CL: Cardiolipin; GGA: Geranylgeranoic acid; LPL: Lysophospholipids; PC: Phosphatidylcholine; PE: Phosphatidylethanolamine; PI: Phosphatidylinositol; PS: Phosphatidylserine; TLC: Thin-layer chromatography; ZAA: Zaragozic acid A.

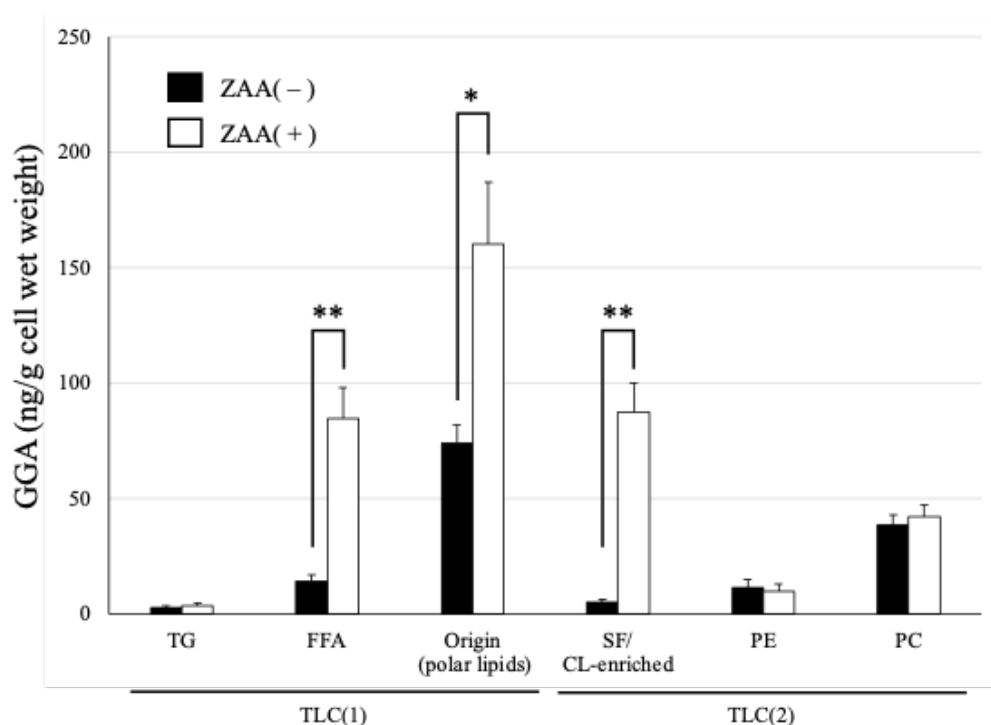


Figure 2. Quantitative comparison of GGA recovery from selected lipid fractions in vehicle- and ZAA-treated HuH-7 cells. HuH-7 cells were treated with vehicle or 2.5 μ M ZAA for 48 h. GGA was quantified by LC-MS/MS after alkaline hydrolysis of TLC-scraped fractions. Black bars indicate vehicle-treated cells, and white bars indicate ZAA-treated cells. GGA recovery from TLC(1) fractions, including TG, FFA, and origin/polar lipids, and TLC(2) fractions, including solvent-front/CL-enriched, PE, and PC fractions, is shown. Data are expressed as mean $\pm$ SD from three independent experiments. Statistical analysis was performed using Welch's t-test. * $p < 0.05$ and ** $p < 0.01$ versus vehicle-treated cells.

Abbreviations: CL: cardiolipin; FFA: free fatty acid; PC: phosphatidylcholine; PE: phosphatidylethanolamine; SF: solvent front; TG: triacylglycerol; TLC: Thin-layer chromatography; ZAA: Zaragozic acid A.

CL-enriched fraction contained only a small amount of GGA under basal conditions. In contrast, ZAA treatment markedly increased GGA recovery from the CL-enriched fraction, while PC remained relatively unchanged and PE showed only a modest decrease. These findings suggest that increased intracellular GGA availability is accompanied by a marked expansion of a CL-enriched GGA-recoverable pool rather than by a simple uniform increase across all phospholipid fractions.¹

The basal recovery of GGA from PC and PE fractions is compatible with the general concept that membrane phospholipids can serve as reservoirs for bioactive lipid precursors. Classical lipid mediators, such as arachidonic acid-derived prostaglandins and leukotrienes, are stored in membrane phospholipids and released through enzymatic hydrolysis in response to cellular stimuli.³⁵ Although GGA is structurally distinct from polyunsaturated fatty acid-derived lipid mediators, the present data raise the possibility that PC- and PE-associated lipid pools may contribute to the basal storage or handling of endogenous GGA in hepatoma cells.

ZAA treatment increased GGA recovery not only from the polar lipid fraction but also from the FFA fraction. This is consistent with the role of ZAA as an inhibitor of squalene synthase that enhances non-sterol isoprenoid flux and increases endogenous GGA availability. Therefore, ZAA-induced GGA accumulation appears to involve both free GGA and hydrolysable lipid-associated GGA pools. Within the phospholipid-associated fractions, however, the most striking change was the marked increase in GGA recovery from the CL-enriched fraction. This suggests that, when intracellular GGA availability is increased, a substantial portion of recoverable GGA becomes associated with a CL-enriched solvent-front fraction.

The simultaneous increase in the FFA fraction and the polar lipid fraction may indicate that ZAA-induced GGA accumulation is partitioned into more than one intracellular pool. The FFA fraction likely reflects free or FFA-migrating GGA generated under enhanced non-sterol isoprenoid flux,^{1,4} whereas the polar lipid fraction may represent hydrolysable lipid-associated GGA pools. This distinction is important because free

GGA and phospholipid-associated GGA may have different biological implications. Free GGA may be more immediately available for receptor interaction, metabolic conversion, or stress signaling,¹² whereas phospholipid-associated GGA may reflect a storage, buffering, or compartmentalized handling mechanism within cellular membranes. Therefore, the present TLC-based approach provides not only quantitative information on total GGA recovery but also a first approximation of how increased endogenous GGA availability is distributed between free and membrane-associated lipid pools. Although this interpretation requires confirmation by comprehensive lipidomic analysis, it provides a useful working model for future studies examining whether distinct GGA pools have different roles in hepatoma-cell stress responses.

The biological significance of increased GGA recovery from the CL-enriched fraction warrants further investigation. Cardiolipin is closely associated with mitochondrial membrane organization, cristae structure, respiratory chain function, and mitochondrial stress responses.^{28,35} Previous studies have shown that higher-dose ZAA-induced intracellular GGA accumulation and exogenous GGA exposure are associated with hepatoma-cell death responses, including mitochondrial dysfunction and inflammatory cell-death-related phenotypes.^{1,12,14,36} However, these previous observations provide biological context only and do not establish a functional connection with the lipid-fraction changes observed in the present study. The present study used lower ZAA concentrations that do not induce significant cytotoxicity within 48 h, allowing analysis of lipid-fraction distribution under non-cytotoxic high-flux conditions. Mitochondrial function, reactive oxygen species (ROS) generation, cardiolipin remodeling, inflammatory signaling, and cell-death endpoints were not measured concurrently. Therefore, the present data do not establish a causal relationship between GGA recovery from the CL-enriched fraction and hepatoma-cell stress responses. Instead, they provide a hypothesis-generating biochemical observation that warrants functional testing under defined GGA-accumulating or stress conditions.

Several non-mutually exclusive mechanisms may underlie the marked increase in GGA recovery from the CL-enriched fraction after ZAA treatment. The present data do not distinguish specific redistribution of GGA into cardiolipin-related pools from broader ZAA-induced remodeling of mitochondrial lipids, altered phospholipid turnover, or nonspecific accumulation of isoprenoid metabolites. One possibility is that basal PC/PE-associated fractions represent major sites of GGA handling, whereas increased GGA availability after ZAA treatment is accompanied by enhanced recovery from the

CL-enriched fraction. Alternatively, ZAA-induced changes in phospholipid abundance, acyl-chain remodeling, or mitochondrial membrane composition may indirectly alter the recovery of GGA among TLC fractions.^{37,38} The relatively unchanged PC recovery and modest reduction in PE recovery are consistent with a prominent increase in GGA recovery from the CL-enriched fraction; however, they do not establish direct transfer of GGA from PC/PE to CL or regulated incorporation into cardiolipin.^{39,40}

These findings also raise the possibility that phospholipid remodeling enzymes participate in the redistribution or retention of GGA. PC and PE remodeling is regulated in part through Lands' cycle-related lysophospholipid acyltransferases, including LPCATs and LPEAT-related enzymes,^{37,38,41} whereas cardiolipin remodeling is regulated by enzymes such as tafazzin and ALCAT1.⁴²⁻⁴⁵ If GGA is incorporated into or retained within hydrolysable phospholipid-associated pools, these remodeling enzymes may influence the distribution, retention, or mobilization of GGA under basal and high-flux conditions. Future studies using genetic or pharmacological modulation of LPCATs, LPEAT-related enzymes, tafazzin, ALCAT1, and related lipid-remodeling pathways will be useful for determining whether the increase in CL-enriched GGA recovery reflects regulated lipid remodeling or secondary redistribution caused by altered isoprenoid metabolism.

4.1. Limitations

First, this study used TLC-based lipid fractionation, and complete chemical purity of each scraped fraction was not confirmed by comprehensive lipidomic profiling.⁴⁶⁻⁵¹ In the primary phospholipid TLC system, the solvent-front fraction was operationally designated as CL-enriched because an authentic CL standard migrated to this region. Supplementary CL-resolving TLC analysis further showed GGA recovery from a fraction co-migrating with the authentic CL standard. Nevertheless, these TLC-based observations do not exclude the presence of co-migrating lipid species and do not identify GGA-containing cardiolipin molecular species. Accordingly, the present data support recovery of hydrolysable GGA from a CL-enriched or CL-associated TLC fraction, rather than definitive structural assignment of GGA to cardiolipin. High-resolution lipidomic analysis of intact molecular species will be required to resolve this issue. Second, GGA was quantified only after alkaline hydrolysis of TLC-scraped material. Therefore, the present workflow does not establish covalent esterification of GGA to phospholipids and cannot distinguish esterified GGA from non-covalently associated, silica-retained, or co-extracted GGA. Accordingly, the term "hydrolysable GGA-recoverable pool" is used operationally to describe

the material from which GGA was recovered after alkaline hydrolysis. Third, no fraction-specific internal standard, spike-recovery experiment, or formal mass-balance validation was performed. Therefore, the sum of TLC(2) fractions cannot be interpreted as a quantitative recovery assessment of the TLC(1) origin fraction. Losses and variability during re-extraction from the TLC(1) origin, the second TLC separation, silica scraping, alkaline hydrolysis, and subsequent extraction may affect the apparent distribution and mass balance between sequential TLC procedures. Fourth, external calibration with authentic GGA was used without a stable isotope-labeled internal standard. Thus, fraction-specific matrix effects, ion suppression, silica-dependent recovery, hydrolysis efficiency, and extraction losses were not independently assessed or corrected. The reported values should therefore be interpreted as comparative GGA recovery under a common analytical workflow rather than as fully matrix-corrected absolute quantification across distinct TLC fractions. Fifth, the statistical analysis was based on three independent experiments per condition. Normality and variance homogeneity were not formally assessed, no adjustment for multiple comparisons was applied, and formal effect-size confidence intervals were not calculated. Although Welch's *t*-test does not assume equal variances, the resulting *p*-values should be interpreted as exploratory and require confirmation in larger independent datasets. Sixth, this study was performed in a single hepatoma cell line. Seventh, total phospholipid abundance, cardiolipin molecular composition, phospholipid turnover, and the chemical form of GGA were not measured. Therefore, the present design cannot distinguish specific redistribution of GGA into cardiolipin-related pools from broader ZAA-induced lipid remodeling or nonspecific accumulation of isoprenoid metabolites. Eighth, mitochondrial function, ROS generation, cardiolipin remodeling, inflammatory signaling, and cell-death assays were not performed concurrently. Therefore, no functional or causal relationship between GGA recovery from CL-enriched fractions and hepatoma-cell stress responses can be inferred from the present study. Thus, the functional significance of GGA redistribution should be addressed in future studies.

Taken together, the present findings provide a basis for future studies on the intracellular handling of endogenous GGA. The observation that basal GGA was mainly recovered from PC/PE fractions, whereas ZAA treatment markedly increased recovery from the CL-enriched fraction, suggests that GGA distribution may be dynamically altered according to intracellular isoprenoid flux. This shift may represent a lipid-fraction signature linking endogenous GGA metabolism to mitochondrial lipid remodeling^{28,43} and hepatoma-cell vulnerability^{3,13,14}

although direct functional validation is required. Further studies combining high-resolution lipidomics, structural identification of GGA-containing molecular species, mitochondrial functional assays, cell-death analyses, and modulation of phospholipid remodeling enzymes will be necessary to determine whether increased recovery of GGA from CL-enriched lipid pools contributes to anticancer-relevant stress responses in hepatoma cells.⁴

5. Conclusion

This study provides an initial characterization of the lipid-fraction distribution of endogenous GGA in HuH-7 hepatoma cells. Under basal conditions, GGA was mainly recovered from polar lipid fractions, with predominant recovery from PC and, to a lesser extent, PE fractions. ZAA treatment increased GGA recovery in both the FFA and polar lipid fractions, and the most prominent change within phospholipid-containing fractions was a marked increase in the solvent-front/CL-enriched fraction. Supplementary CL-resolving TLC analysis further supported the recovery of GGA from a CL-associated fraction. These findings indicate that increased intracellular GGA availability is associated with the expansion of a CL-associated, hydrolysable GGA-recoverable pool. Although the chemical form of GGA in these fractions and their functional significance require further investigation, the observed lipid-fraction shift provides a rationale for examining potential relationships between endogenous GGA metabolism, mitochondrial lipid remodeling, and hepatoma-cell stress responses. Future studies combining intact-species lipidomics with mitochondrial and cell-death assays will be required to determine whether GGA recovery from CL-associated fractions has functional relevance to the tumor-suppressive actions of GGA in hepatoma cells.

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Conflict of interest

The author declares no conflicts of interest.

Author contributions

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

Data are available from the corresponding author upon reasonable request.

References

- Shidoji Y, Tabata Y. Unequivocal evidence for endogenous geranylgeranoic acid biosynthesized from mevalonate in mammalian cells. *J Lipid Res.* 2019;60:579-593.
doi: 10.1194/jlr.M090548
- Tabata Y. Quantitative Screening of Geranylgeranoic Acid in Selected Plant-Based Foods Using LC/MS/MS. *Front Nutr.* 2025;12:1652270.
doi: 10.3389/fnut.2025.1652270
- Shidoji Y, Ogawa H. Natural occurrence of cancer-preventive geranylgeranoic acid in medicinal herbs. *J Lipid Res.* 2004;45:1092-1103.
doi: 10.1194/jlr.M300502-JLR200
- Verdaguer IB, Hernández A, Torres S, et al. Prenols and prenoic acids in metabolism, disease, and aging. *J Lipid Res.* 2026;67:101009.
doi: 10.1016/j.jlr.2026.101009
- Tabata Y. Geranylgeranoic acid and the MAOB-CYP3A4 axis: a metabolic shift underlying age-related liver cancer risk. *Front Aging.* 2025;6:1680031.
doi: 10.3389/fragi.2025.1680031
- Mitake M, Shidoji Y. Geranylgeraniol oxidase activity involved in oxidative formation of geranylgeranoic acid in human hepatoma cells. *Biomed Res (Tokyo).* 2012;33:15-24.
doi: 10.2220/biomedres.33.15
- Araki H, Shidoji Y, Yamada Y, Moriwaki H, Muto Y. Retinoid Agonist Activities of Synthetic Geranyl Geranoic Acid Derivatives. *Biochem Biophys Res Commun.* 1995;209:66-72.
doi: 10.1006/bbrc.1995.1471
- Muto Y, Moriwaki H, Ninomiya M, et al. Prevention of second primary tumors by an acyclic retinoid, polyprenoic acid, in patients with hepatocellular carcinoma. Hepatoma Prevention Study Group. *N Engl J Med.* 1996;334:1561-1567.
doi: 10.1056/NEJM199606133342402
- Muto Y, Moriwaki H, Saito A. Prevention of second primary tumors by an acyclic retinoid in patients with hepatocellular carcinoma. *N Engl J Med.* 1999;340:1046-1047.
doi: 10.1056/NEJM199904013401315
- Omori M, Watanabe M, Shidoji Y, Moriwaki H, Muto Y. Inhibitory effects of a timely dosing of acyclic retinoid on the development of spontaneous hepatomas in C3H/HeNCrj mice. *Recent Adv Gastroenterol Carcinog.* 1996;1:523-526.
- Omori M, Shidoji Y, Moriwaki H. Inhibition of Spontaneous Hepatocarcinogenesis by 4,5-Didehydrogeranylgeranoic Acid: Effects of Small-Dose and Infrequent Administration. *Int J Transl Med.* 2023;3:487-495.
doi: 10.3390/ijtm3040034
- Yabuta S, Shidoji Y. TLR4-mediated pyroptosis in human hepatoma-derived HuH-7 cells induced by a branched-chain polyunsaturated fatty acid, geranylgeranoic acid. *Biosci Rep.* 2020;40:BSR20194118.
doi: 10.1042/BSR20194118
- Shidoji Y. Geranylgeranoic acid, a bioactive and endogenous fatty acid in mammals: a review. *J Lipid Res.* 2023;64(7):100396.
doi: 10.1016/j.jlr.2023.100396
- Shidoji Y. Induction of Hepatoma Cell Pyroptosis by Endogenous Lipid Geranylgeranoic Acid-A Comparison with Palmitic Acid and Retinoic Acid. *Cells.* 2024;13:809.
doi: 10.3390/cells13100809
- Shidoji Y, Okamoto K, Muto Y, Komura S, Ohishi N, Yagi K. Prevention of geranylgeranoic acid-induced apoptosis by phospholipid hydroperoxide glutathione peroxidase gene. *J Cell Biochem.* 2006;97:178-187.
doi: 10.1002/jcb.20627
- Tabata Y. Endogenous and dietary geranylgeranoic acid: A mini review on its role in hepatic tumor suppression. *Eurasian J Med Oncol.* 2025;9:356-361.
doi: 10.36922/EJMO025330352
- Ha NT, Lee CH. Roles of Farnesyl-Diphosphate Farnesyltransferase 1 in Tumour and Tumour Microenvironments. *Cells.* 2020;9:2352.
doi: 10.3390/cells9112352
- Muehlebach ME, Holstein SA. Geranylgeranyl diphosphate synthase: Role in human health, disease and potential therapeutic target. *Clin Transl Med.* 2023;13:e1167.
doi: 10.1002/ctm2.1167
- Park J, Matralis AN, Berghuis AM, Tsantrizos YS. Human isoprenoid synthase enzymes as therapeutic targets. *Front Chem.* 2014;2:50.
doi: 10.3389/fchem.2014.00050
- Hooff GP, Volmer DA, Wood WG, Müller WE, Eckert GP. Isoprenoid quantitation in human brain tissue. A validated HPLC-fluorescence detection method for endogenous farnesyl- (FPP) and geranylgeranylpyrophosphate (GGPP). *Anal Bioanal Chem.* 2008;392:673-680.
doi: 10.1007/s00216-008-2306-3
- Popják G, Cornforth RH, Clifford K. Inhibition of cholesterol

- biosynthesis by farnesoic acid and its analogues. *Lancet*. 1960;275:1270-1273.
doi: 10.1016/S0140-6736(60)92252-2
22. Tabata Y. Monoamine oxidase B as a context-dependent metabolic switch in hepatocellular carcinoma. *Eurasian J Med Oncol*. 2026;10:025520546.
doi: 10.36922/EJMO025520546
23. Ortiz-Placín C, Castillejo-Rufo A, Estarás M, González A. Membrane Lipid Derivatives: Roles of Arachidonic Acid and Its Metabolites in Pancreatic Physiology and Pathophysiology. *Molecules*. 2023;28:4316.
doi: 10.3390/molecules28114316
24. Bergstroem S, Danielsson H, Samuelsson B. The enzymatic formation of prostaglandin e2 from arachidonic acid prostaglandins and related factors 32. *Biochim Biophys Acta*. 1964;90:207-210.
doi: 10.1016/0304-4165(64)90145-x
25. Akerele OA, Cheema SK. A diet enriched in longer chain omega-3 fatty acids reduced placental inflammatory cytokines and improved fetal sustainability of C57BL/6 mice. *Prostaglandins Leukot Essent Fatty Acids*. 2018;137:43-51.
doi: 10.1016/j.plefa.2018.08.002
26. Shapiro H, Singer P, Ariel A. Beyond the classic eicosanoids: Peripherally-acting oxygenated metabolites of polyunsaturated fatty acids mediate pain associated with tissue injury and inflammation. *Prostaglandins Leukot Essent Fatty Acids*. 2016;111:45-61.
doi: 10.1016/j.plefa.2016.03.001
27. Pizzuto M, Pelegrin P. Cardiolipin in Immune Signaling and Cell Death. *Trends Cell Biol*. 2020;30:892-903.
doi: 10.1016/j.tcb.2020.09.004
28. Li ZZ, Xiao HX, Hu JJ, *et al*. The Mechanisms and Implications of Cardiolipin in the Regulation of Cell Death. *Cell Biochem Funct*. 2025;43:e70066.
doi: 10.1002/cbf.70066
29. Fuentes JM, Morcillo P. The Role of Cardiolipin in Mitochondrial Function and Neurodegenerative Diseases. *Cells*. 2024;13:609.
doi: 10.3390/cells13070609
30. Paradies G, Paradies V, Ruggiero FM, Petrosillo G. Role of Cardiolipin in Mitochondrial Function and Dynamics in Health and Disease: Molecular and Pharmacological Aspects. *Cells*. 2019;8:728.
doi: 10.3390/cells8070728
31. Zhou J, Zhu D, Wang Y, *et al*. Mitochondrial stress orchestrates chromatin remodeling and longevity via phosphoregulation of the NuRD component LIN-40. *Sci China Life Sci*. 2025;68:3340-3352.
doi: 10.1007/s11427-025-2954-3
32. Claypool SM, Koehler CM. The complexity of cardiolipin in health and disease. *Trends Biochem Sci*. 2012;37:32-41.
doi: 10.1016/j.tibs.2011.09.003
33. Dudek J. Role of Cardiolipin in Mitochondrial Signaling Pathways. *Front Cell Dev Biol*. 2017;5:90.
doi: 10.3389/fcell.2017.00090
34. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem*. 1957;226(1):497-509.
doi: 10.1016/S0021-9258(18)64849-5
35. Carney OS, Harris K, Santizo M, *et al*. A review of disorders of cardiolipin metabolism: Pathophysiology, clinical presentation and future directions. *Mol Genet Metab*. 2025;145:109184.
doi: 10.1016/j.yimgme.2025.109184
36. Iwao C, Shidoji Y. Induction of nuclear translocation of mutant cytoplasmic p53 by geranylgeranoic acid in a human hepatoma cell line. *Sci Rep*. 2014;4:4419.
doi: 10.1038/srep04419
37. Wang B, Tontonoz P. Phospholipid Remodeling in Physiology and Disease. *Annu Rev Physiol*. 2019;81:165-188.
doi: 10.1146/annurev-physiol-020518-114444
38. Roggero A, Toyama MH, Sousa SF. Lands' Cycle at the Crossroads: Phospholipid Remodelling, Oxidative Stress, Cellular Toxicity, and Therapeutic Targeting. *ACS Pharmacol Transl Sci*. 2025;8:3729-3756.
doi: 10.1021/acsptsci.5c00482
39. Zhang H, Yu F, Tian Z, Jia D. Cardiolipin Remodeling in Cardiovascular Diseases: Implication for Mitochondrial Dysfunction. *Acta Physiol (Oxf)*. 2025;241:e70073.
doi: 10.1111/apha.70073
40. Di K, Hu Y, Sun H, Meng T, Han T, Qie R. Cardiolipin remodelling in mitochondrial therapeutics: translational evidence chains from elamipretide to emerging strategies. *Front Physiol*. 2026;17:1813119.
doi: 10.3389/fphys.2026.1813119
41. Korbecki J, Bosiacki M, Pilarczyk M, *et al*. Phospholipid Acyltransferases: Characterization and Involvement of the Enzymes in Metabolic and Cancer Diseases. *Cancers (Basel)*. 2024;16:2115.
doi: 10.3390/cancers16112115
42. Cao J, Liu Y, Lockwood J, Burn P, Shi Y. A novel cardiolipin-remodeling pathway revealed by a gene encoding an endoplasmic reticulum-associated acyl-CoA:lysocardiolipin acyltransferase (ALCAT1) in mouse. *J Biol Chem*.

- 2004;279:31727-31734.
doi: 10.1074/jbc.M402930200
43. Schlame M. Cardiolipin remodeling and the function of tafazzin. *Biochim Biophys Acta*. 2013;1831:582-588.
doi: 10.1016/j.bbalip.2012.11.007
44. Bione S, D'Adamo P, Maestrini E, Gedeon AK, Bolhuis PA, Toniolo D. A novel X-linked gene, G4.5, is responsible for Barth syndrome. *Nat Genet*. 1996;12:385-389.
doi: 10.1038/ng0496-385
45. Schlame M, Greenberg ML. Biosynthesis, remodeling and turnover of mitochondrial cardiolipin. *Biochim Biophys Acta Mol Cell Biol Lipids*. 2017;1862:3-7.
doi: 10.1016/j.bbalip.2016.08.010
46. Tague ED, Woodall BM, Harp JR, Farmer AT, Fozo EM, Campagna SR. Expanding lipidomics coverage: effective ultra performance liquid chromatography-high resolution mass spectrometer methods for detection and quantitation of cardiolipin, phosphatidylglycerol, and lysyl-phosphatidylglycerol. *Metabolomics*. 2019;15:53.
doi: 10.1007/s11306-019-1512-7
47. Gao F, McDaniel J, Chen EY, *et al*. Adapted MS/MSALL Shotgun Lipidomics Approach for Analysis of Cardiolipin Molecular Species. *Lipids*. 2018;53:133-142.
doi: 10.1002/lipd.12004
48. Bird SS, Marur VR, Sniatynski MJ, Greenberg HK, Kristal BS. Lipidomics profiling by high-resolution LC-MS and high-energy collisional dissociation fragmentation: focus on characterization of mitochondrial cardiolipins and monolysocardiolipins. *Anal Chem*. 2011;83:940-949.
doi: 10.1021/ac102598u
49. Han X, Gross RW. The foundations and development of lipidomics. *J Lipid Res*. 2021;63:100164.
doi: 10.1016/j.jlr.2021.100164
50. Bautista JS, Falabella M, Flannery PJ, *et al*. Advances in methods to analyse cardiolipin and their clinical applications. *Trends Analyt Chem*. 2022;157:116808.
doi: 10.1016/j.trac.2022.116808
51. Han X, Yang K, Yang J, Cheng H, Gross RW. Shotgun lipidomics of cardiolipin molecular species in lipid extracts of biological samples. *J Lipid Res*. 2006;47:864-879.
doi: 10.1194/jlr.D500044-JLR200

Appendix

Table A1. Supplementary CL-resolving TLC analysis of GGA recovery from cardiolipin-related fractions in ZAA-treated HuH-7 cells

TLC system	Fraction	GGA (ng/g cell wet weight)
CL-resolving TLC	CL	92.6 ± 17.5
	PE	11.7 ± 4.6
	MLCL	N.D.
	LPE	N.D.

Notes: Polar lipid extracts from HuH-7 cells treated with 2.5 µM ZAA for 48 h were separated using a CL-resolving TLC solvent system. Because this TLC system did not resolve PC, these data were used as supplementary validation of CL-associated GGA recovery and were not included in the main phospholipid-fraction distribution analysis. Values are expressed as mean ± SD from three independent experiments. GGA was quantified by LC-MS/MS after alkaline hydrolysis of TLC-scraped fractions.

Abbreviations: CL: Cardiolipin; GGA: Geranylgeranoic acid; LPE: Lysophosphatidylethanolamine; MLCL: Monolysocardiolipin; N.D.: Not detected; PE: Phosphatidylethanolamine; TLC: Thin-layer chromatography; ZAA: Zaragozic acid A.