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Inhibition of lysine-specific demethylase 1 by the acyclic diterpenoid geranylgeranoic acid and its derivatives

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ABSTRACT

Lysine-specific demethylase 1 (LSD1) is upregulated in many cancers, especially neuroblastoma. We set out to explore whether geranylgeranoic acid (GGA) inhibits LSD1 activity by using recombinant human LSD1. GGA inhibited LSD1 activity with IC_{50} similar to that of the clinically used drug tranylcypromine. In human neuroblastoma SH-SY5Y cells, GGA induced *NTRK2* gene expression alongside upregulation of histone H3 with dimethylated lysine-4 in the regulatory regions of the *NTRK2* gene. Dihydrogenation of GGA reinforced the LSD1-inhibitory effect in a position-dependent manner. The inhibitory effects of dihydro-derivatives of GGA on recombinant LSD1 strongly correlated with the induction of *NTRK2* gene expression in SH-SY5Y cells. These data demonstrate for the first time the efficient LSD1-inhibitor activity of GGA and its derivatives, providing a novel prospect of preventing cancer onset by using GGA to regulate epigenetic modification.

1. Introduction

Epigenetic genome regulatory mechanisms are essential for appropriate development and cellular differentiation in mammals. Furthermore, epigenetic alterations are key causes of aberrant gene function leading to cancer [1,2]. Global hypomethylation of genomic DNA is commonly observed in cancerous cells, leading to genomic instability and activation of growth-promoting genes. Conversely, site-specific hypermethylation of promoter region CpG islands is also typical of cancers, causing silencing of tumor-suppressor genes. Additionally, increased histone deacetylase activity in tumor cells results in a global loss of acetylated histones and subsequent nucleosome remodeling.

Histone lysine methylation is also extensively involved in nucleosome remodeling and gene expression. Methylation is reversibly regulated by histone lysine methyltransferases and histone lysine demethylases (KDMs). Although a number of KDMs have so far been identified, lysine-specific demethylase 1 (LSD1; also known as KDM1, BHC110 or AOF2) has received increasing attention since its identification in 2004 [3]. LSD1 is capable of removing dimethyl and monomethyl groups on lysine 4 of histone H3 (H3K4me2 and H3K4me1, respectively), as well as methyl groups on non-histone proteins such as a tumor suppressor p53 and DNA methyltransferase 1 [4]. Overexpression of LSD1 is frequently observed in prostate, breast, and bladder cancers, and especially neuroblastoma, where it correlates directly with adverse clinical outcome and inversely with differentiation [5]. Thus, LSD1 inhibitors are of clinical interest for their anticancer role as well as their potential application in other human diseases that exhibit deregulated gene expression.

LSD1 is a flavin adenine dinucleotide (FAD)-containing enzyme belonging to the amine oxidase superfamily [6]. The structural homology between LSD1 and monoamine oxidase-B (MAOB), a clinically validated pharmacological target, suggests that LSD1 is a druggable target. Indeed, screening of known MAO inhibitors has uncovered sub-millimolar LSD1 inhibitors among which the best known is the antidepressant drug tranylcypromine (*trans*-2-phenylcyclopropylamine). The drug acts as an irreversible inhibitor forming a covalent adduct with the FAD cofactor of LSD1 with a *Ki* value of 242 μM [7]. Besides the antidepressant, the terpene *trans*, *trans*-farnesol, a component of tobacco smoke, is well known as a potent, reversible, specific inhibitor of mammalian MAOB [8,9]. *Ki* values of farnesol for the inhibition of human, baboon, monkey, dog, rat, and mouse liver MAOB are within the range of 0.5–5 μM. Although three-dimensional structures of MAOB-farnesol [9], MAOB-tranylcypromine [10], and LSD1-tranylcypromine [11] complexes have been determined to sub-molecular resolution, no published structure of the LSD1-farnesol complex is available. Furthermore, while several candidate LSD1 inhibitor compounds have been synthesized based on the structures of LSD1 and MAOB in complexes with the antidepressant tranylcypromine, limited

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studies on the structure-activity relationship of farnesol analogs with human LSD1 have been reported to date.

Recently, we reported that all-*trans* geranylgeranoic acid (GGA), a natural acyclic diterpenoid found in medicinal herbs [12], inhibited proliferation of human neuroblastoma-derived SH-SY5Y cells and induced a neuronal phenotype via upregulation of tropomyosin-related kinase receptor B (*TrkB* or neurotrophin receptor kinase-2, *NTRK2*) gene expression [13]. Epigenetic modifications of H3K4me3 in the promoter regions of the *NTRK2* gene in NeuN positive neuronal cells have also been reported [14]. LSD1 is frequently upregulated in clinical neuroblastoma samples as well as in neuroblastoma cell lines including SH-SY5Y. While LSD1 cannot act directly on tri-methylated histones, JARID1B (a demethylase that specifically removes methyl groups from H3K4me3) and LSD1 act in a sequential and coordinated manner to demethylate H3K4me3 [15]. Combining these observations led us to speculate that LSD1-dependent downregulation of H3K4me3 occurs in the promoter regions of the *NTRK2* gene in SH-SY5Y cells. Indeed, Schulte and colleagues found that inhibition of LSD1 by tranylcypromine led to accumulation of H3K4me2 in SH-SY5Y cells and prevented SH-SY5Y xenograft growth in mice [5].

Here, we report that GGA and its dihydro-derivatives are potent inhibitors of recombinant human LSD1. Furthermore, these compounds induce *NTRK2* gene expression via upregulation of H3K4me2 in the putative promoter regions of the *NTRK2* gene in human neuroblastoma SH-SY5Y cells.

2. Materials and methods

2.1. Materials

Farnesol, *trans*-2-phenylcyclopropylamine hydrochloride (2-PCPA), geraniol, and geranylgeraniol were purchased from Sigma–Aldrich (St. Louis, MO, USA). Farnesoic acid, farnesyl amine, farnesyl amide, geranic acid, and geranylgeranoic acid (GGA) were kind gifts from Kuraray (Okayama, Japan). (*R*)- and (*S*)-2,3-dihydroGGAs were synthesized as follows: ω,E,E,E- and ω,E,E,Z-geranylgeranoates were obtained by the condensation of farnesyl acetone with triethylphosphonoacetate followed by silica gel column chromatography for purification. Each molecule was then subjected to (*S*)-p-tol-BINAP reduction followed by hydrolysis with KOH [16]. 6,7-DihydroGGA was prepared as follows: Geranylacetone was condensed with triethylphosphonoacetate to yield an unsaturated ester, which was reduced by bis(2-methoxyethoxy)-aluminum hydride in the presence of CuBr to afford the saturated ester. The ester was converted into the corresponding 6,7-dihydroGGA ester according to a previously reported method [17]. Basic hydrolysis

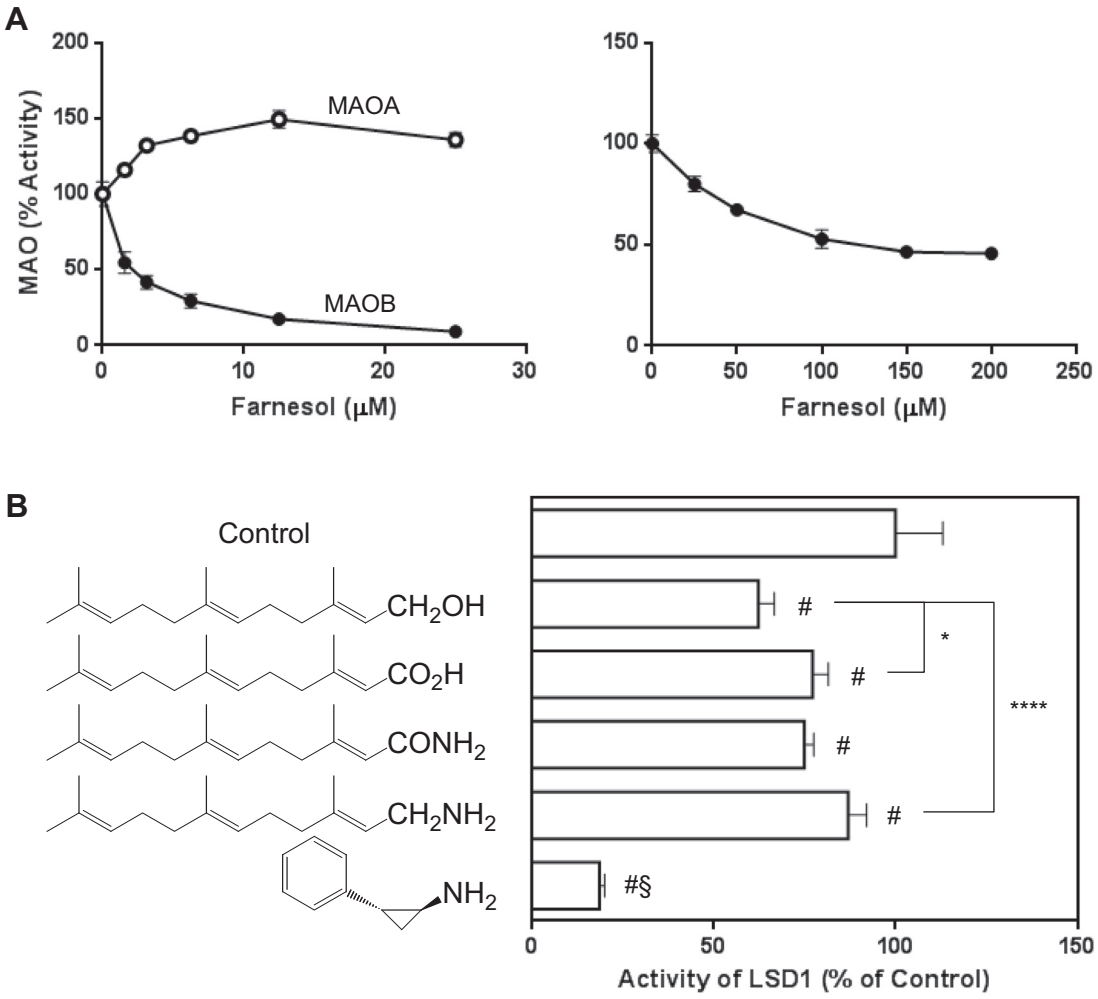

Fig. 1. Inhibition of recombinant human LSD1 by farnesol. (A) Left panel: Inhibitory effect of farnesol on MAO activity. Right panel: Farnesol inhibited recombinant human LSD1 activity. (B) LSD1 inhibitory effects of farnesol derivatives were assessed at 100 μM. 2-PCPA was used as a positive control. Results were presented as the mean ± SD and analyzed by Tukey's multiple comparisons test, # significant vs. control, § significant vs. farnesol derivatives, $^{*}p < 0.05$, $^{****}p < 0.0001$.

of the ester produced the 6,7-dihydroGGA. Similarly, 10,11-dihydro- and 14,15-dihydroGGA were prepared from citronellyl bromide and 6-methyl-5-hepten-2-one, respectively.

2.2. Monoamine oxidase (MAO) activity analysis

The inhibitory effect of farnesol on MAO activity was measured using MAO-Glo™ Assay (Promega, Madison, WI, USA) and recombinant human MAOA and MAOB (Sigma–Aldrich). Farnesol was dissolved in ethanol to produce a 20-fold diluted solution with MAO Reaction Buffer (Promega) and pre-incubated with enzymes (5 mU) for 1 h on ice. Following oxidization of MAO substrate (40 μM to assay MAOA or 4 μM for MAOB) at 37 °C for 1 h, the methyl ester luciferin, which is produced by the action of MAO on MAO substrate, was reacted with esterase and luciferase for 20 min at room temperature. The produced light was measured with CentroXS^3 LB960 (Berthold Japan K.K., Tokyo, Japan).

2.3. Lysine-specific demethylase 1 (LSD1) inhibitory analysis

Determination of inhibitory activity against LSD1 was performed with the LSD1 Inhibitor Screening Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA). The enzyme reaction was monitored by fluorescence on channel 1 of a LightCycler 1.5 (Roche Diagnostics K.K., Tokyo, Japan) for 30 min.

2.4. Cell culture and treatment

Human neuroblastoma SH-SY5Y cells were inoculated at 1×10^4 cells/cm^2 and cultured in D-MEM (4500 mg/L glucose, Wako Pure Chemicals, Osaka, Japan) containing 10% heat-inactivated fetal bovine serum (FBS; Thermo Scientific Hyclone, Yokohama, Japan) for 2 d. Thereafter, the medium was replaced with fresh D-MEM containing 10% FBS and any relevant test compound at the required concentration. Ethanol (0.1%, v/v) was used as a vehicle control.

2.5. Reverse transcription and quantitative polymerase chain reaction (RT-qPCR)

After 48 h treatment, total RNA was isolated from cell cultures using the High Pure RNA Isolation Kit (Roche Diagnostics). For cDNA synthesis, Transcriptor Universal cDNA Master (Roche Diagnostics) was used according to the manufacturer's instruction. Real-time PCR was performed using LightCycler 1.5 and DyNAmo™ Capillary SYBR™ Green qPCR Kit (Thermo Fisher Scientific, Waltham, MA, USA). Primer sequences used in this study are presented

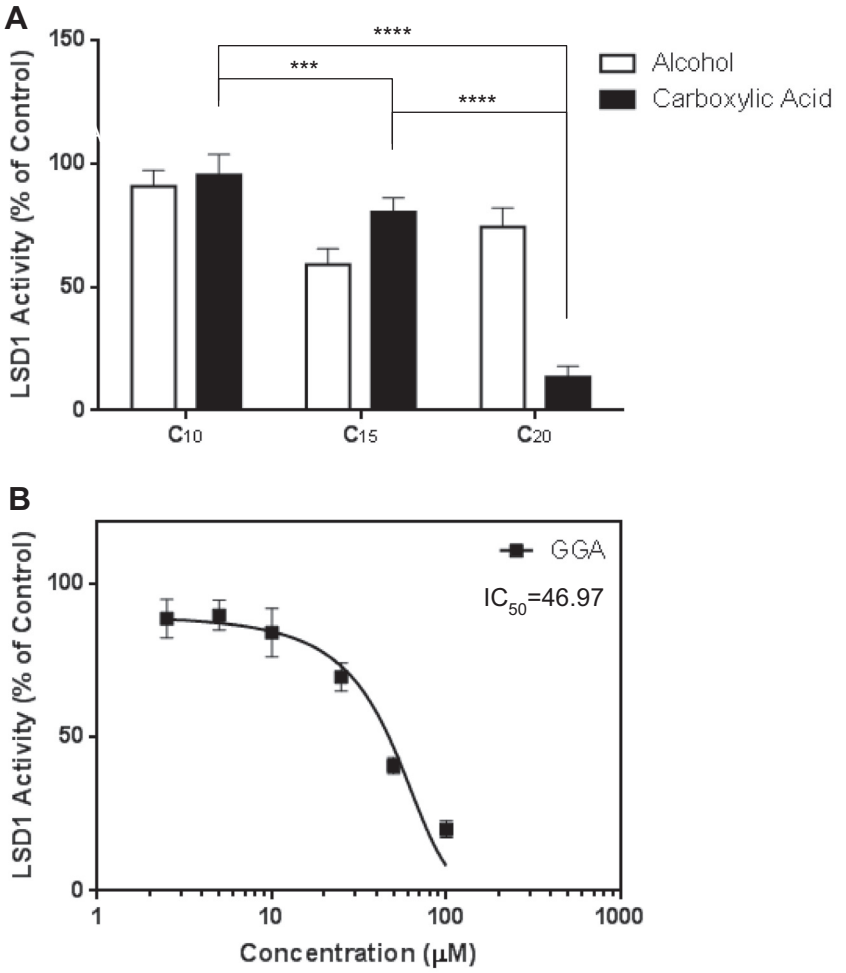

Fig. 2. Isoprenoid chain length-dependent inhibition of LSD1 activity by polyprenoic acids. (A) LSD1-inhibitory effect of different chain-length polyprenoids. Each inhibitor was used at a concentration of 100 μM. Data are expressed as the mean ± SD ($n = 5$) and analyzed by Tukey's multiple comparisons test, $^{***}p < 0.001$, $^{****}p < 0.0001$. (B) Dose-dependent inhibition of LSD1 activity by GGA. Data are expressed as mean ± SD.

in Supplementary Table S1. Gene expression levels were analyzed using the $2^{-\Delta\Delta Ct}$ method.

2.6. Chromatin immunoprecipitation assay (ChIP)

Cross-linked chromatin digestion and immunoprecipitation were carried out with SimpleChIP™ Enzymatic Chromatin IP Kit (Cell Signaling Technology, Danvers, MA, USA) and di-methyl lysine 4 anti-histone H3 (anti-H3K4me2) antibody (Cell Signaling Technology, #9725) according to the manufacturer's instruction. DNA was purified with QIAquick® PCR Purification Kit (QIAGEN, Tokyo, Japan) and quantified by real-time PCR with specific primers listed in Supplementary Table S1.

3. Results

3.1. Inhibition of LSD1 activity by farnesol

First, farnesol was confirmed as a micromolar inhibitor of human MAOB. Farnesol inhibited recombinant MAOB enzyme with an IC_{50} value of 2 μM (Fig. 1A, left panel). This IC_{50} is close to the reported *Ki* value for farnesol with human MAOB [8]. Farnesol did not inhibit MAOA, rather a slight activation was observed with increasing concentrations of farnesol. Fig. 1A, right panel, shows that farnesol also inhibited recombinant human LSD1 activity in a dose-dependent manner with IC_{50} of approximately 120 μM when the first 21 amino acid-containing peptide of the N-terminal tail of histone H3 was used as substrate. This indicates that farnesol is also a weak inhibitor for human LSD1.

Conversion of the terminal functional group of farnesol from alcohol to either a carboxyl, amide, or amine group did not enhance inhibitor activity at 100 μM, whereas the standard inhibitor tranylcypromine efficiently inhibited LSD1 activity at the same concentration (Fig. 1B).

3.2. Effects of isoprenoid chain length on LSD1 inhibitory activity

When the terminal functional group of polyisoprenoids was alcohol, the number of isoprene units in each molecule, from two (C_{10}) to four (C_{20}), did not affect LSD1 inhibitory activity (Fig. 2A). Conversely, when a carboxyl terminal group was present, increasing the number of isoprene unit from two (C_{10}) to four (C_{20}) significantly increased LSD1 inhibitory activity. Consequently, C_{20}-GGA was by far the most potent inhibitor of LSD1 tested (Fig. 2A). The IC_{50} of C_{20}-GGA was 47 μM (Fig. 2B), and the mode of the inhibition was non-competitive (Supplementary Fig. S1).

3.3. Effects of dihydrogenation on LSD1-inhibitory activity of GGA

Dihydrogenation of isoprene units on GGA reinforced its inhibitory activity, dependent on the site of hydrogenation

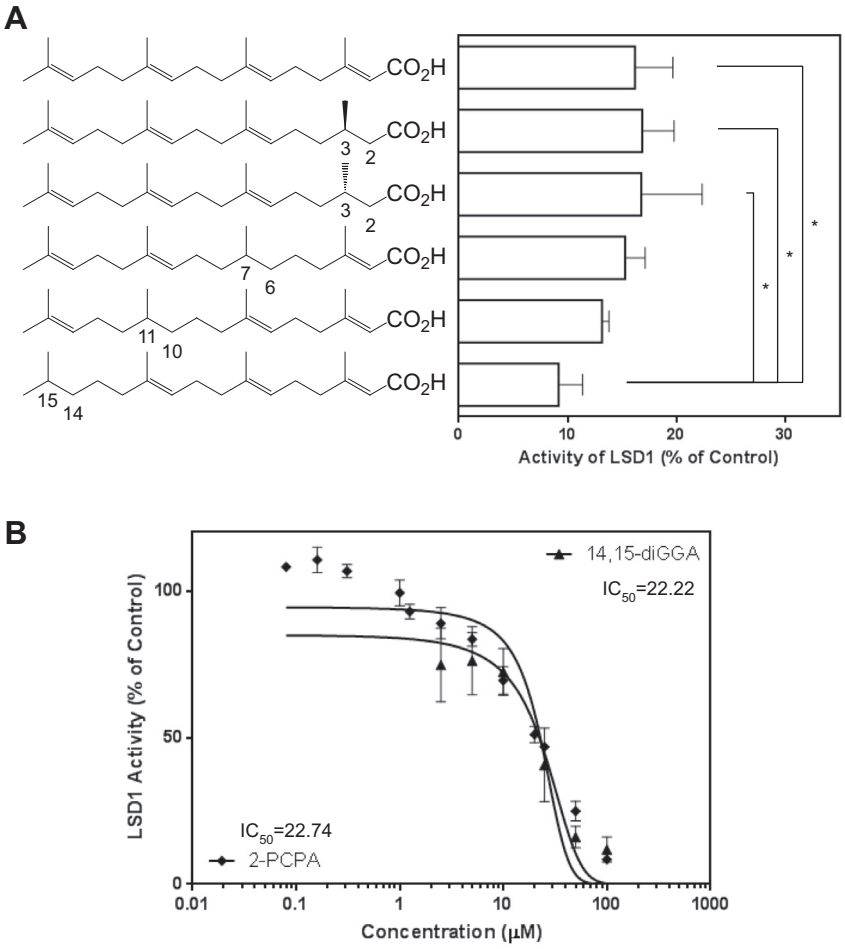

Fig. 3. Effect of dihydrogenation on the LSD1-inhibitory activity of GGA. (A) Dihydro-GGAs (100 μM each) were pre-incubated with hLSD1 and LSD1 enzyme activity was measured. Data are presented as mean ± SD and analyzed by Tukey's multiple comparisons test, $^{*}p < 0.05$. (B) LSD1-inhibitory effects of 14,15-dihydroGGA and 2-PCPA at the indicated concentrations. Results are presented as the mean ± SD.

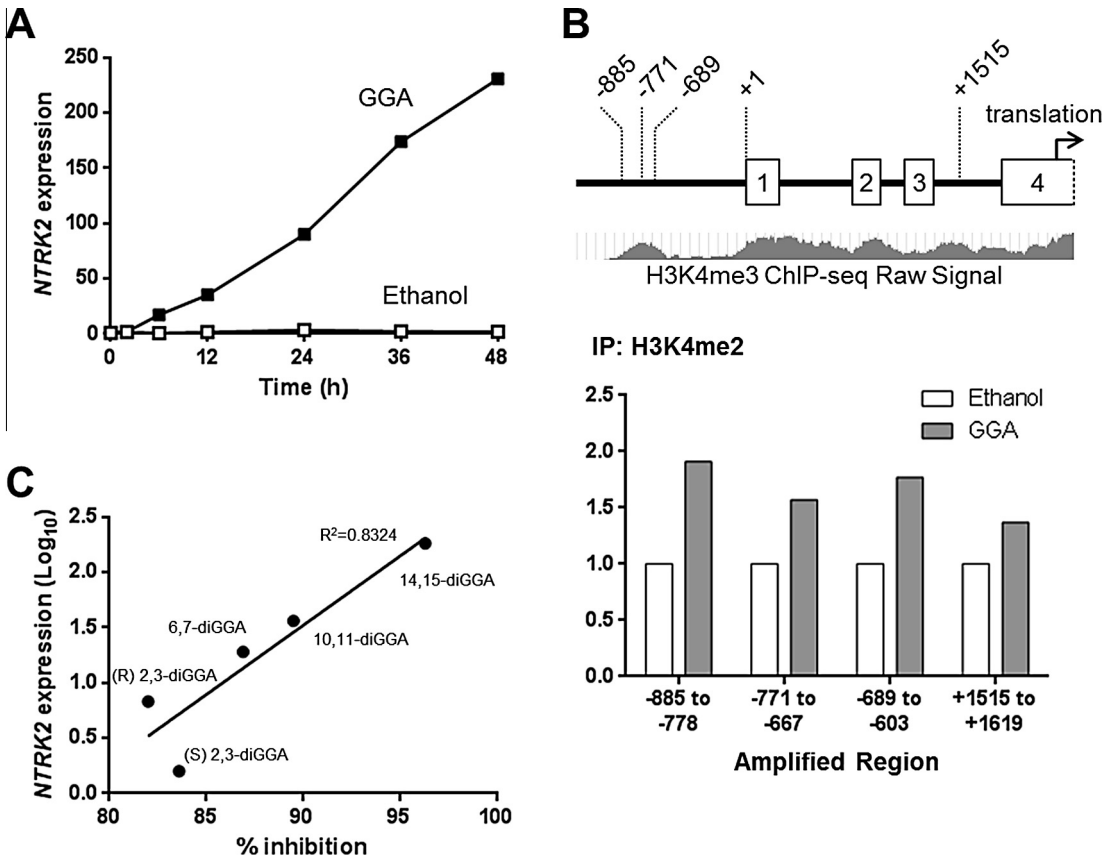

Fig. 4. LSD1-inhibition is associated with induction of *NTRK2* gene expression. (A) GGA induced *NTRK2* gene expression in a time-dependent manner. SH-SY5Y cells were treated with 10 μM GGA for the indicated times. (B, upper panel) Schematic representation of the upstream portion of the *NTRK2* gene with H3K4me3 status. +1 indicates the transcriptional start site. H3K4me3 ChIP-seq Raw Signal image was adopted from UCSC Genome Browser. (B, lower panel) GGA treatment induced de-methylated H3K4 bound to promoter regions of the *NTRK2* gene. Cells were treated with 10 μM GGA for 2 h, and ChIP assay was performed. The data were normalized to input DNA. (C) Correlation between LSD1 inhibition and *NTRK2* expression levels. *NTRK2* mRNA levels were analyzed by RT-qPCR after 48 h treatment with 10 μM dihydro-GGAs.

(Fig. 3A). From most to least potent, the LSD1-inhibitory activity of GGA dihydro-derivatives was as follows: 14,15- > 10,11- > 6,7- > (*S*)2,3- > (*R*)2,3-dihydroGGA. This suggests that a steric structure of a straight tetra-isoprenoid chain is important for inhibition of hLSD1 activity. The most potent inhibitor, 14,15-dihydroGGA, had an IC_{50} of 22 μM, comparable to that of 2-PCPA (23 μM; Fig. 3B).

3.4. Upregulation of H3K4me2 bound to the promoter region of the NTRK2 gene by GGA

We recently reported that GGA upregulates cellular levels of three major *NTRK2* messenger RNA splice variants, as well as two variant forms (145 and 95 kD) of the NTRK2 protein in human neuroblastoma SH-SY5Y cells [13]. In the present study, GGA-induced upregulation of the full-length mRNA of the *NTRK2* gene was confirmed in Fig. 4A.

ChIP-seq technology revealed neuron-specific upregulation of H3K4me3 bound to putative P1 (from −885 to −689) and P2 (around +1515) promoter regions of the *NTRK2* gene as depicted in Fig. 4B, upper panel [14]. Therefore, we conducted H3K4me2 ChIP assay with PCR primers targeted to the P1 and P2 promoter regions. GGA treatment significantly increased the cellular levels of H3K4me2 bound to both promoter regions of the *NTRK2* gene (Fig. 4B).

The recombinant-hLSD1-inhibitory effects of several dihydro-derivatives of GGA correlated with their ability to induce *NTRK2* gene expression in SH-SY5Y cells (Fig. 4C). This suggests that dihydroGGA-induced inhibition of LSD1 activity may be responsible for the drug-induced upregulation of *NTRK2* gene expression.

4. Discussion

The present study examines the inhibitory effect of the acyclic diterpenoid GGA on the epigenetic regulator enzyme LSD1. The rationale for conducting this work stems from the observations that the antidepressant tranylcypromine, an efficient MAO inhibitor, can also inhibit LSD1, a member of the FAD-containing amine oxidase superfamily and that farnesol, an acyclic sesquiterpenoid found in tobacco smoke, is a competitive inhibitor of human MAOB. In this context, farnesol was expected to inhibit LSD1 activity, which it did in a dose-dependent manner (Fig. 1A). Furthermore, data presented in this study clearly demonstrates that GGA is a more potent inhibitor of human LSD1 than farnesol and that this inhibition occurs in a non-competitive fashion.

Interestingly, dihydro-derivatives of GGA were more potent inhibitors of LSD1 than GGA. Of the dihydro-derivatives tested, 14,15-dihydroGGA was the most potent inhibitor with an IC_{50} of 22 μM, comparable with that (23 μM) of the clinically-used inhibitor tranylcypromine. Although tranylcypromine is used for treatment of depression and is proven to be safe, it is a purely synthetic chemical and may be impractical to take for prolonged

periods of time to prevent carcinogenesis. In contrast, the natural diterpenoid GGA and 14,15-dihydroGGA are branched chain fatty acids, with which human cells are familiar as components of the mevalonate-pathway. In fact, GGA is a natural diterpenoid found in several medicinal herbs [12] and oral administration of 4,5-dide-hydroGGA (600 mg/day) for 1 year has been proven to significantly prevent primary hepatoma-eradicated patients from developing recurrent primary hepatoma [18]. GGA has also been scientifically proven to be safe without any side effects [19]. Therefore, we speculate that human cells should tolerate prolonged administration of GGA or 14,15-dihydroGGA at their effective doses.

Recently, we reported that GGA suppressed cellular proliferation and induced neuronal differentiation and upregulation of neuron-specific *NTRK2* gene expression in human neuroblastoma SH-SY5Y cells [13]. This cell line is known to strongly express LSD1. Schulte and colleagues found that tranylcypromine-mediated inhibition of LSD1 reprograms the transcriptome of SH-SY5Y cells and administration of the drug inhibits neuroblastoma xenograft growth in mice [5]. We propose that GGA is able to inhibit LSD1 activity resulting in global upregulation of H3K4me2 levels within cells. In support of this, GGA treatment increased cellular H3K4me2 levels present around the P1 and P2 promoter regions of the *NTRK2* gene in human neuroblastoma SH-SY5Y cells (Fig. 4). Such histone modifications, in general, cause transcriptional activation of the gene. In the present study, a time-dependent activation of *NTRK2* gene expression was indeed demonstrated after GGA treatment (Fig. 4A). As neuronal differentiation involves increased cellular levels of H3K4me3 around the P1 and P2 promoter regions of the *NTRK2* gene, GGA treatment altered neuroblastoma cells from an undifferentiated to a differentiated state via upregulation of H3K4me2 levels around the P1 and P2 regions of the *NTRK2* gene. This relationship is further supported by a significant association between the LSD1-inhibitory effects and the *NTRK2* gene-upregulating effects of the dihydro-GGAs (Fig. 4C).

The initiation and progression of cancer, traditionally seen as a genetic disease, is now known to involve epigenetic abnormalities along with genetic alterations. A new terminology "histone onco-modifications" has been proposed to describe the post-translational modifications of histones that have been linked to carcinogenesis. LSD1, which belongs to a flavin-dependent amine oxidase superfamily, consists of a growing number of transcriptional complexes that are implicated in carcinogenesis [20]. Hence, there has been an increased effort to identify or design LSD1 inhibitors that could function as antitumor epigenetic therapeutic agents [6]. Here, LSD1 inhibition with small molecule inhibitors resulted in growth inhibition of tumor cells in vitro and an increase in global H3K4me2 methylation.

The present study clearly demonstrates that GGA and its dihydro-derivatives represent most promising cancer-preventive epigenetic therapeutic agents targeting LSD1, and further work to this end is warranted.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.12.144.

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