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Identification of Chaulmoogric Acid as a Small Molecule Activator of Protein Phosphatase 5

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Abstract Protein phosphatase 5 (PP5) is an important protein phosphatase that is abundantly expressed in the central nervous system. Recent studies showed that PP5 activity in the neocortex from patients with Alzheimer's disease (AD) is decreased significantly, suggesting that small molecule PP5 activator may have therapeutic potential for AD. We performed a biochemical screening for PP5 activators with the microsource compound library. Chaulmoogric acid was identified to be an effective activator with EC_{50} value of 134.5 μM. Importantly, results from circular dichroism (CD) and limited proteolysis study showed that chaulmoogric acid binds to a region of tetratricopeptide repeat (TPR) domain of PP5 resulting in complete loss of helical contents. These results demonstrate a different mechanism of action from that of arachidonic acid, a known activator for PP5 dephosphorylation activity. Synergistic activation of PP5 enzymatic activity was also observed with combined application of both compounds at relatively low concentrations. Therefore, further structure activity relationship study of chaulmoogric acid may facilitate the discovery of small molecules that can synergize with endogenous arachidonic acid for PP5 activation.

Keywords PP5 · TPR · Arachidonic acid · Chaulmoogric acid · High-throughput screening

Introduction

Temporal and spatial regulation of protein phosphorylation is vital to all eukaryotic cells. Although protein kinases have been the major focus of pharmaceutical industry, protein phosphatases are starting to attract more attention in recent years [1]. Current views suggest that protein phosphatases are tightly and dynamically regulated. Importantly, studies from transgenic mice-expressing protein phosphatases or knockout mice have already provided starting points for the development of drugs [2, 3]. PP5 is a newly identified member of the

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PPP family of serine/threonine protein phosphatases [4–6]. Previous reports showed that PP5 participates in several stress-activated cellular signaling pathways including DNA damage-induced apoptosis [3], oxidative stress [7], and UV- or γ-irradiation [8, 9]. However, all these results were from studies employing siRNA, antisense, and over-expression of PP5. Few pharmacologically relevant substrates have been identified in the cellular environment [10].

Tau is one of the microtubule-associated proteins in neurons with the known activity of stimulating and maintaining the assembly and stability of microtubules [11]. The hyper-phosphorylation of tau is proposed to account for its loss of function, gain of toxicity, and aggregation to paired helical filaments. The latter forms neurofibrillary tangles observed in AD and several other tauopathies [12]. Evidence from recent studies strongly suggested that PP5 associates with microtubules and dephosphorylates tau in vitro [10, 13, 14]. Tau phosphorylated by cAMP-dependent protein kinase and glycogen synthase kinase-3β, two of the major tau kinases, could be dephosphorylated by PP5 both in vitro and in vivo. Liu et al. also reported that PP5 dephosphorylates tau at all 12 AD-associated abnormal phosphorylation sites [10]. Expression of active PP5 in PC-12 cells decreased phosphorylation of tau. Furthermore, the activity of PP5 was found to be reduced by 20% in the AD neocortex [10]. These results suggest that PP5 may serve as a physiological phosphatase of tau, particularly in the brain. Hyperphosphorylation of tau in AD may be reversed by activating PP5.

PP5 is composed of two structurally distinct domains: the N-terminal tetratricopeptide repeat (TPR) domain and the C-terminal phosphatase domain. A subdomain of 13 residues forms a short two-turn α-helix (αJ) which is connected to the phosphatase domain [15, 16]. In the crystal structure of human PP5, Yang and his colleagues demonstrated the detailed molecular mechanism by which PP5 autoinhibits its own phosphatase activity [17]. Intramolecular interactions between the turns connecting three tandem TPR motifs create a contiguous ridge that inserts into the catalytic channel, thus, blocking potential substrates from accessing the catalytic center [17]. The C-terminal αJ helix further stabilizes the interaction between the TPR and the phosphatase domain through hydrophobic contacts with the TPR domain. The fact that removal of either the TPR domain or the 13 C-terminal residues by proteolysis leads to a constitutively active phosphatase aligns well with evidences from the 3D structure.

Small molecule activators of PP5 phosphatase activity, including polyunsaturated fatty acids, have been reported previously [18, 19]. Arachidonic acid stimulates full-length PP5 with EC_{50} values between 50 and 125 μM [18, 19], which is much greater than its physiological concentration. However, long-chain fatty acyl-CoA esters activate PP5 at physiological concentrations of ~1 μM [20], suggesting that PP5 activation can be regulated by endogenous long acyl chain molecules. It has been proposed that the CoA moiety can enhance the overall solubility of the long-chain fatty acids in aqueous cytoplasm [20, 21]. Evidence obtained using far UV circular dichroism has shown that fatty acid can significantly reduce the α-helical content of the TPR domain while increasing its melting temperature [17]. Therefore, long-chain fatty acyl-CoA activates PP5 by inducing conformational changes in TPR domain such that the TPR can no longer form stable contacts with the catalytic site of the phosphatase domain. No other small molecules have been identified so far that can induce the phosphatase activity of this important enzyme.

In the present study, we established a biochemical screen for small molecule activators of PP5. The rationale for our study is twofold: (1) a novel activator with different chemical structure can be a candidate for structure–activity relationship (SAR) studies and lead generation for neurodegenerative diseases particularly tauopathies; (2) small chemicals identified in the screen can be also used as research tools for better understanding of the

molecular regulation of PP5 activity both in vitro and in vivo. Our results demonstrate that chaulmoogric acid is a bona fide activator with a different mode of action to that of arachidonic acid.

Materials and Methods

Materials

All chemicals used in this study are of analytical grade. Para-nitrophenylphosphate (pNPP) was purchased from Fisher Scientific (PA, USA). Arachidonic acid and trifluoroethanol were purchased from Sigma Chemicals (MO, USA). The Microsource library and chaulmoogric acid were purchased from Microsource Discovery Systems (CT, USA).

Cloning, Expression, and Purification of PP5

Recombinant PP5 (16–499) and TPR (16–147) were expressed as N-terminal gluthathione-*S*-transferase (GST)-tagged fusion proteins in BL21 (DE3; Invitrogen, CA). PP5 and TPR domain were first purified by affinity chromatography using a Glutathione Sepharose Fast Flow column (GE Healthcare) followed by anion exchange chromatography using a Q Sepharose Fast Flow column (PP5) or cation exchange chromatography using a MonoS column (TPR). In both instances, the GST-tag was cleaved on-column by PreScission Protease (GE Healthcare, NJ), and purification was carried out according to manufacturer's instructions. Purified PP5 and TPR were aliquoted and stored at −80 °C.

Assay for PP5 Activity and EC_{50} Determination

Recombinant PP5 was assayed with pNPP as previously described [19] with the following modifications; 50 μl of PP5 in reaction buffer (50 mM Tris-HCl, pH 7.5, 5 mM dithiothreitol (DTT), 0.1 mM ethylenediaminetetracetic acid (EDTA), and 0.4 mg/ml bovine serum albumin) was added to each well of a 96-well half area plate (Corning Incorporated, NY) followed by the addition of 20 μl of arachidonic acid in sterile water. The mixture was incubated at 23 °C for 30 min. Thirty microliters of pNPP in reaction buffer was added to initiate the reaction, which was allowed to proceed at 23 °C for 60 min. Control reactions were assayed without enzyme or arachidonic acid. Assays were terminated by the addition of 10 μl of 1 M sodium hydroxide (NaOH) solution and absorbance at 405 nm was measured using the EnVision 2104 Multilabel Reader (Perkin Elmer, MA, USA). The final concentrations of PP5, arachidonic acid, and pNPP in each 100 μl reaction were 10.2 nM, 3.9–250 μM, and 11 mM, respectively.

High-Throughput Screening of PP5 Activators and Hit Confirmation

Approximately 1,120 compounds from the Microsource library were screened for activators of PP5, and hits were confirmed by determining their EC_{50} values. The pNPP assay was automated for high throughput screening using a Sciclone ALH3000 Workstation (Caliper Life Sciences, MA) according to the previous publication [22]. Compound dilution plates for screening (final screening concentration 25 μM, 1:400-fold dilution) and EC_{50} determination (final concentration range 3.9 μM–1 mM, 1:200-fold dilution) were prepared in polypropylene V-bottom 96-well plates (Corning Incorporated). In both cases, the first

and last columns were designated for the positive control (100 μM arachidonic acid) and the negative control (DMSO only), respectively. Absorbance was measured, and fold activation was calculated as described in the previous section. EC_{50} values were calculated using the GraphPad Prism software.

CD Spectroscopy

Circular dichroism experiments were carried out on a Jasco J-715 spectropolarimeter in buffer containing 10 mM Tris (pH 7.0) and 1 mM DTT. The far-UV CD spectra were recorded using a 1-mm path length cuvette for PP5 at the concentration of 20 μM with scanning range between 190–270 nm (0.5 nm/interval) at 10 °C. Arachidonic acid or chaulmoogric acid was dissolved in trifluoroethanol (TFE) to reduce the background at 222 nm. These two compounds were then added to 20 μM PP5 solution with a final concentration of 200 μM and incubated at room temperature for 10 min. Concentrations of the proteins were determined spectroscopically by absorbance at 280 nm.

Limited Proteolysis

Limited proteolysis of the TPR domain was performed on ice in 40-μl reactions consisting of 4 μg of purified protein, 200 μM of arachidonic acid, or chaulmoogric acid dissolved in TFE and 0.4 μl of 0.05 mg/ml proteinase K in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 1 mM DTT. There were no significant changes observed when TFE alone was used on protease K digestion of TPR domain (data not shown). Arachidonic acid or chaulmoogric acid was incubated with TPR at 23 °C for 30 min before the addition of proteinase K. The reaction was allowed to proceed for 30 min before being terminated by the addition of 2 μl of 0.1 M phenylmethylsulphonylfluoride. Samples were frozen on dry ice until analysis by 4~12% Bis-Tris sodium dodecyl sulfate gel electrophoresis.

Compound Synergistic Study

To determine synergy between arachidonic acid and chaulmoogric acid in activating PP5, 10–200 μM of arachidonic acid or chaulmoogric acid was co-incubated with 7.8–250 μM of chaulmoogric acid or arachidonic acid respectively in a 100-μl reaction, which was assayed for PP5 activity as described in the previous section.

Results

Compound Screening with pNPP Assay

The pNPP release assay [19] for measuring PP5 phosphatase activity was adapted for high throughput screening. When PP5 was activated with 40 μM of arachidonic acid, the Km of pNPP was calculated to be 6.8 mM (data not shown), which is close to the Km value reported previously [19]. Based on our enzyme titration results, 10.2 nM of PP5 was used for compound screening with a pNPP concentration of 11 mM (data not shown). Using arachidonic acid as a positive control, we automated the pNPP assay using a Sciclone ALH3000 (Caliper Life Science, Hopkinton, MA) with a high Z' factor of 0.7, a tight coefficient of variation (CV) value of 6.9%, and a large ratio of signal versus background of 7.4.

Thus, the pNPP assay was considered to have been successfully converted into a robotic 96-well microplate format. A high-throughput screen was performed at a final concentration of 25 μM. As shown in Fig. 1A, PP5 treated with 100 μM of arachidonic acid exhibited an average of 7.45-fold increase in phosphatase activity. Four compounds were able to activate PP5 by at least 1.4-fold (Fig. 1A) at 25 μM.

We further performed a concentration dependent study on these four primary hits to confirm their activities in an EC_{50} format. Chaulmoogric acid (Compound 1 in Fig. 1A), a small molecule with an extended 18-carbon chain structure (Table 1), exhibited an EC_{50} value of 134.50±0.17 μM (Fig. 1B). As a positive control, the EC_{50} value for arachidonic acid was calculated at 77.59±0.05 μM (Fig. 1B), a value that is within the range reported previously [18, 19]. We also observed that fold activation decreased at concentrations of arachidonic acid greater than 250 μM, similar to what was previously observed [19]. Although chaulmoogric acid has a slightly higher EC_{50} value than arachidonic acid, both compounds produced similar fold activation indexes (16~17-fold) at their maximal induction level (Fig. 1B). Unlike chaulmoogric acid, the other three compounds (Compound 2~4 in Fig. 1A) did not show a strong dose-dependent behavior and were eliminated from further follow-up studies.

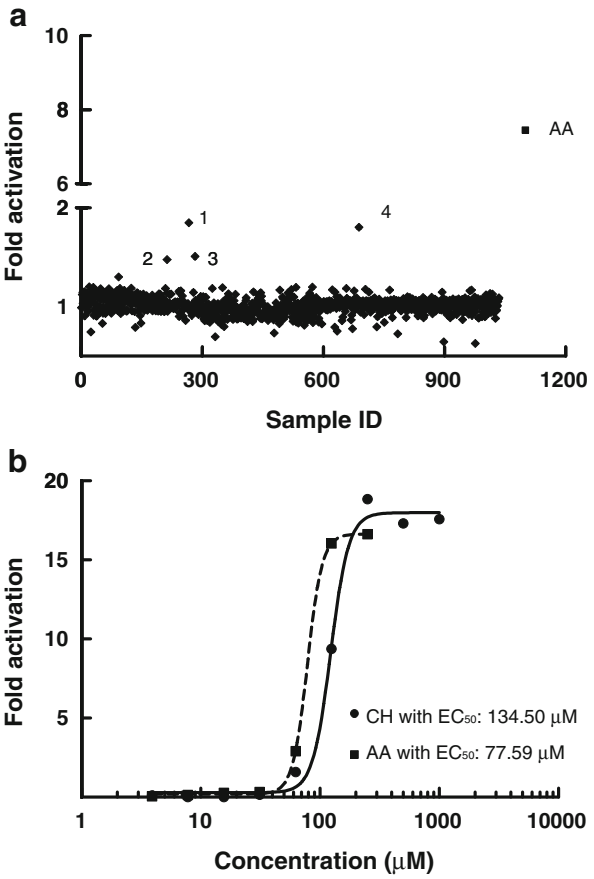
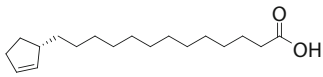
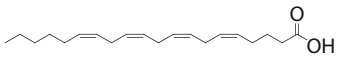


Fig. 1 **A** Screening for PP5 activators with 1,120 Microsource compounds (*filled diamonds*) at 25 μM on a Sciclone ALH3000; 100 μM of arachidonic acid (*AA*; *filled squares*) was used as a positive control. pNPP assay was performed as described in "Materials and Methods." Fold activation was calculated by normalizing absorbance values of compound-treated samples at 405 nm to that of DMSO-treated controls. **B** EC_{50} evaluation of arachidonic acid (*filled squares*) and chaulmoogric acid (*filled circles*). Each data point represents the average of three determinations

Table 1 Compound structure and molecular weight.

Name	MW	Structure
Chaulmoogric acid	280.5	
Arachidonic acid	326.0	

Compound-Induced Conformational Changes in the TPR Domain

It is of particular interest to know whether chaulmoogric acid activates PP5 activity in the same fashion as arachidonic acid. A 15% reduction of α-helical content was observed in the TPR domain in the presence of 20 μM arachidonoyl-CoA [17]. Additionally, the melting temperature was recorded to be 5 °C higher in arachidonoyl-CoA-treated TPR as compared to protein alone. We documented a 26% reduction in far UV CD signal at 222 nm when purified TPR domain was incubated with 200 μM arachidonic acid (Fig. 2A). Most interestingly, chaulmoogric acid disrupted TPR α-elical structure in a completely different manner than arachidonic acid with a CD spectrum exhibiting β-sheet-like configuration (Fig. 2B). Limited proteolysis was also performed to monitor the structural consequence of compound binding of the TPR domain of PP5. In order to maximize the stability of TPR structure and to make sure that the observed differences reflect properties of the substrate rather than specificity of the protease, we chose to use a nonspecific protease (protease K) and carried out our experiments at 0 °C. As shown in Fig. 3, the TPR domain retained a 15 kDa fragment after treatment of proteinase K, suggesting a relatively stable folding architecture formed by 3.5 TPR repeats. Upon the introduction of arachidonic acid, the proteinase K-resistant fragment diminished and was replaced by a 10 kDa molecular weight band. In contrast, ~40% of TPR still showed proteolytic resistance in the presence of chaulmoogric acid along with the generation of a similar 10 kDa proteolytic product (Fig. 3). It should be noted that neither arachidonic acid nor chaulmoogric acid at 200 μM has any effects on the activity of protease K itself (data no shown). This different digestion profile supports the notion that arachidonic acid and chaulmoogric acid induce different local structural changes and/or spatial arrangements of TPR and convert autoinhibited PP5 into its bioactive form.

Synergistic Effects of Arachidonic Acid and Chaulmoogric Acid

Figure 4 shows the effects of coadministration of arachidonic acid and chaulmoogric acid on PP5 activation. When treated with 31.25 μM or lower concentrations of chaulmoogric acid alone, PP5 exhibited no phosphatase activity (Fig. 4A). Interestingly, more than 2~20-fold increase of PP5 activity was observed when 10~200 μM arachidonic acid was applied to the sample. Similarly, 10~200 μM of chaulmoogric acid can also activate PP5 activity by 2~19.8-fold in the concentration range (31.25 μM or lower) that arachidonic acid alone does not activate PP5 (Fig. 4B). Therefore, both arachidonic acid and chaulmoogric acid demonstrate synergistic effects at relatively low concentrations.

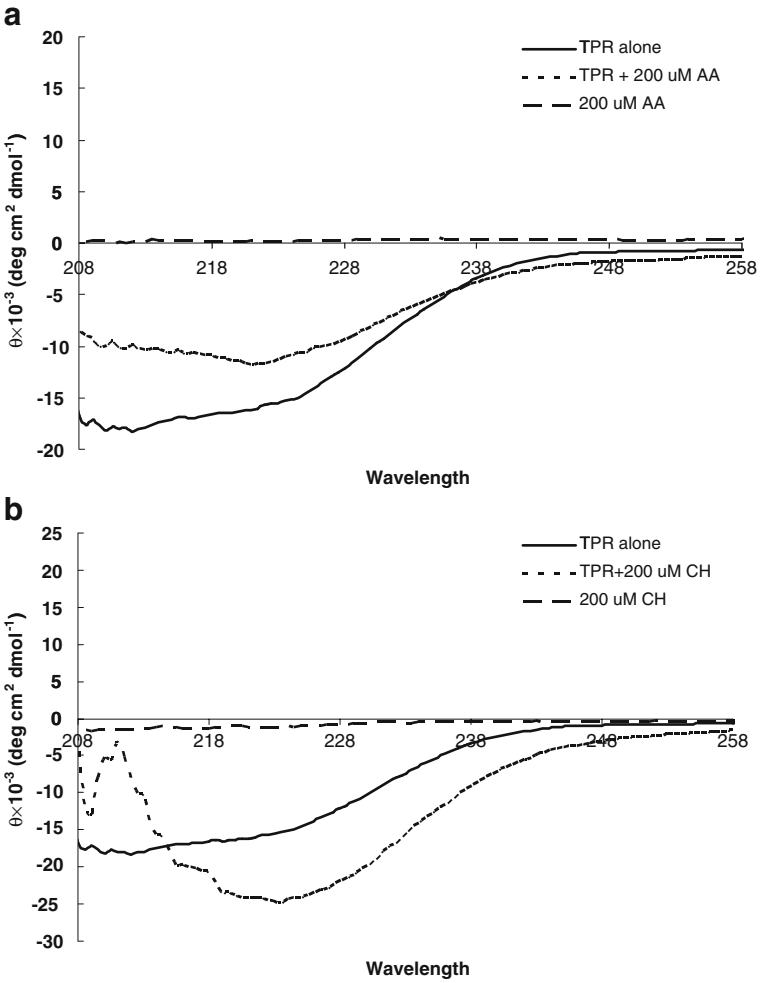


Fig. 2 **A** Far UV CD spectral analysis of TPR (*solid line*), 200 μM AA in trifluoroethanol (*dashed line*) and TPR with 200 μM arachidonic acid (*broken line*). **B** Far UV spectral analysis of TPR (*solid line*), 200 μM chaulmoogric acid (CH) in trifluoroethanol (*dashed line*) and TPR with 200 μM chaulmoogric acid (*broken line*). Each data point is the average of three tested samples

Discussion

In this study, chaulmoogric acid was identified as an activator of PP5 through a high throughput screen. Chaulmoogric acid is one of the predominant fatty acids in chaulmoogra oil isolated from the seeds of tropical Flacourtiaceae [23]. This 18 carbon long-chain molecule is a cyclopentenyl fatty acid that is esterified to glycerol. Structurally, chaulmoogric acid contains a unique monounsaturated five-membered ring distal to the carboxylic acid (Table 1). Ramsey and Chinkers reported that long-chain fatty acids with 16 carbons or more are required for optimal activation of PP5 [20]. One possible explanation

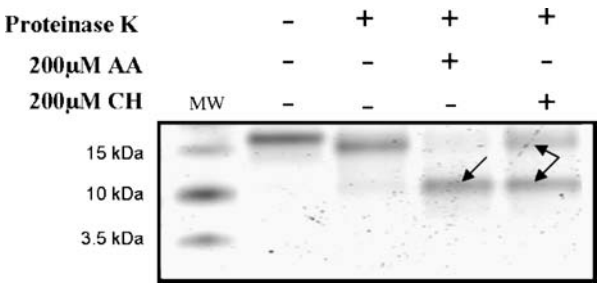


Fig. 3 Limited proteolysis of TPR domain in the absence or presence of 200 μM of arachidonic acid (*AA*) or chaulmoogric acid (*CH*). Treatment with proteinase K was allowed to proceed for 30 min on ice before gel electrophoresis. *Arrows* show the proteolytic product when *AA* or *CH* was added to the protein samples. The molecular weight standard (*MW*) is shown on the *left*

for this phenomenon can be derived by analogy to the structure of the complex that Hop forms with terminal peptides from HSP90/HSP70, in which the peptides lie in an extended conformation [24]. Therefore, the activity of the 18-carbon chaulmoogric acid is consistent with the previous observation but represents a different chemical structure. Importantly, chaulmoogra oil and some related preparations were widely used in the treatment of human leprosy for many years. An earlier study showed that chaulmoogric acid can inhibit the multiplication of *Mycobacterium leprae* in a mouse model of footpad infection [23]. The discovery of a novel mechanism of PP5 activation makes chaulmoogric acid a good starting point for SAR study. Data from our synergy study (Fig. 4) provide a proof-of-concept that we may use potent chemical derivatives from chaulmoogric acid to activate PP5 activity.

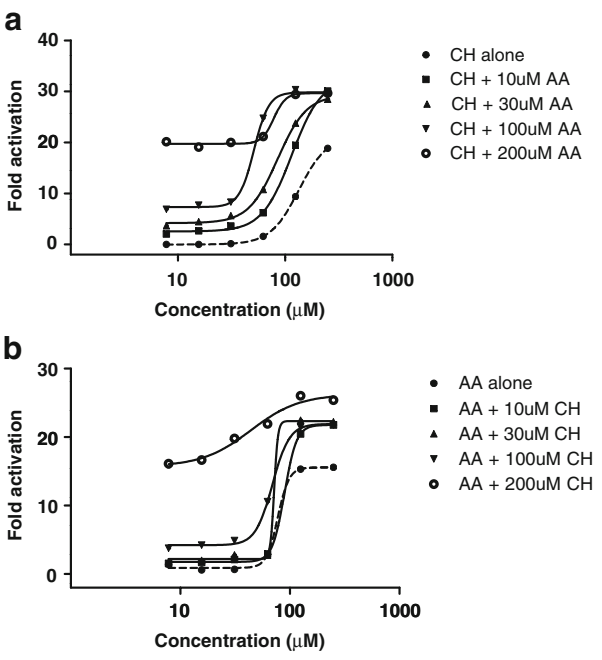


Fig. 4 Experiments to test compound synergy in the activation of full-length PP5 were performed by combining **A** 10–200 μM chaulmoogric acid (*CH*) with 7.8–250 μM arachidonic acid (*AA*) or **B** 10–200 μM AA with 7.8–250 μM CH. Arachidonic acid or chaulmoogric acid alone was used as a control (*broken line*). Each data point represents the mean value from triplicate samples

Table 2 EC_{50} measurement of compound synergistic study.

CH+X μM AA	EC50 (μM)	AA+X μM CH	EC50 (μM)
CH alone	134.50±0.17	AA alone	77.59±0.05
+ 10 μM AA	115.50±0.10	+ 10 μM CH	89.20±0.06
+ 30 μM AA	85.23±0.13	+ 30 μM CH	71.81±0.10
+ 100 μM AA	51.34±0.20	+ 100 μM CH	69.23±0.11
+ 200 μM AA	78.44±0.12	+ 200 μM CH	43.88±0.50

EC_{50} value represents the average of three determinations

The in vivo studies of chaulmoogric acid or its chemical derivatives in AD model systems may advance our knowledge in how PP5 regulates the phosphorylation of tau and whether increased activation of endogenous PP5 can inhibit the initiation of neurodegeneration.

Structurally, chaulmoogric acid causes different conformational changes in the TPR domain compared to arachidonic acid. Arachidonyl-CoA, the ester form of arachidonic acid, converts the TPR domain into a more stable conformation with reduced helical content [17]. In contrast, chaulmoogric acid removes the helical contents in the TPR domain (Fig. 2B). The resulting β-sheet-like structure may induce a more dramatic change in the arrangements of secondary structure in the TPR domain. How such a structural change relates to the unusual monounsaturated five-membered ring in chaulmoogric acid remains to be studied. The results from limited proteolysis and synergy study are consistent with the hypothesis that these two compounds may bind to different regions of the TPR domain (Figs. 3 and 4). In a previous study, an allosteric mechanism was proposed based on the flexibility of the recognition mechanism of different TPR domains [24]. It is highly possible that different small molecules bind to local structures distinct from the direct binding interface of the TPR and phosphatase domains rather than direct competition for relief of autoinhibition. Importantly, it has been reported that human plasma levels of free arachidonic acid (nonesterified) vary from 5.8 to 49.3 μM [25]. The concentration range at which chaulmoogric acid can exhibit its synergistic effects is l7-fold lower than its EC_{50} (Fig. 4B). It is, therefore, possible to use relatively low concentrations of chaulmoogric acid to synergize with endogenous arachidonic acid for PP5 activation, which may be of therapeutic significance. Furthermore, addition of chaulmoogric acid was able to increase maximal PP5 activation at saturating concentrations of arachidonic acid (>100 μM) from 15- to 25-fold. Similar results were observed with chaulmoogric acid treatment. The EC_{50} values of compound synergistic study were summarized in Table 2. These results suggest that the two compounds exert synergistic effects, most likely through different binding mechanisms towards the TPR domain. Nevertheless, both arachidonic acid and chaulmoogric acid activate PP5 through unfolding of the TPR structure and, thus, releasing the catalytic domain from inhibition. The study of small molecule activation of PP5 will shed light on how TPR motifs, which occur in more than 150 human genes, mediate protein–protein interactions in different biological pathways [17, 26]. The availability of chaulmoogric acid as another research tool should greatly facilitate these studies.

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