Lipocalin-Type Prostaglandin D Synthase Is a Novel Phytocannabinoid-Binding Protein

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Abstract

Lipocalin-type prostaglandin D synthase (L-PGDS; EC:5.3.99.2) is an enzyme with dual functional roles as a prostaglandin D_2-synthesizing enzyme and as an extracellular transporter for diverse lipophilic compounds in the cerebrospinal fluid (CSF). Transport of hydrophobic endocannabinoids is mediated by serum albumin in the blood and intracellularly by the fatty acid binding proteins, but no analogous transport mechanism has yet been described in CSF. L-PGDS has been reported to promiscuously bind a wide variety of lipophilic ligands and is among the most abundant proteins found in the CSF. Here, we examine the binding of several classes of endogenous and synthetic ligands to L-PGDS. Endocannabinoids exhibited low affinity toward L-PGDS, while cannabinoid metabolites and synthetic cannabinoids displayed higher affinities for L-PGDS. These results indicate that L-PGDS is unlikely to function as a carrier for endocannabinoids in the CSF, but it may bind and transport a subset of cannabinoids.

Keywords

Introduction

The endocannabinoid system regulates diverse biological processes including cognition and pain (Fowler, Naidu, Lichtman, & Onnis, 2009; Jonsson, Holt, & Fowler, 2006; Maccarrone et al., 2015; Schlosburg, Kinsey, & Lichtman, 2009). The endocannabinoids *N*-arachidonoylethanolamine (anandamide, AEA) and 2-arachidonoylglycerol (2-AG) serve as endogenous ligands for cannabinoid receptors (Dalton, Bass, Van Horn, & Howlett, 2009; Pertwee et al., 2010). Endocannabinoid signaling is terminated by cellular uptake followed by intracellular hydrolysis (Glaser, Kaczocha, & Deutsch, 2005; McKinney & Cravatt,

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2005). Because of their inherent hydrophobicity, endocannabinoids require transport through the aqueous cytosol to reach their catabolic enzymes. Intracellular endocannabinoid trafficking is facilitated by fatty acid binding proteins (FABP), a family of small cytosolic proteins that bind and transport hydrophobic lipids including the endocannabinoids (Huang et al., 2016; Kaczocha, Glaser, & Deutsch, 2009). Within the blood, endocannabinoids are primarily transported by binding to serum albumin for which they exhibit high affinity (Bojesen & Hansen, 2003), but lipo-proteins may also play a partial role (Bilgin, Bindila, Graessler, & Shevchenko, 2015; Ruiz, Sanchez, Correnti, Strong, & Ganfornina, 2013).

Endocannabinoids are also released by cells and are present in the human cerebrospinal fluid (CSF) (Azim et al., 2015; Koethe, Giuffrida, et al., 2009; Morgan et al., 2013; Nicholson et al., 2015). Brain endocannabinoids regulate neurological function, and CSF endocannabinoid levels are altered in patients with schizophrenia, chronic pain, and Parkinson's disease (Azim et al., 2015; Giuffrida et al., 2004; Koethe, Giuffrida, et al., 2009; Morgan et al., 2013; Pisani et al., 2010; Sarchielli et al., 2007). Furthermore, endocannabinoids and the structurally related N-acylethanolamine (NAE) and oleoylethanolamide (OEA)—which activates the nuclear peroxisome proliferator-activated receptor alpha—regulate sleep (Murillo-Rodriguez et al., 2016; Pava, Makriyannis, & Lovinger, 2016; Soria-Gomez et al., 2010). Oleamide is a primary fatty acid amide that likewise regulates sleep, and both oleamide and OEA levels are elevated in the CSF after sleep deprivation (Cravatt et al., 1995; Koethe, Schreiber, et al., 2009). Given the fluctuations in endocannabinoid and the related bioactive lipid levels in the CSF of patients, coupled with their hydrophobicity, we hypothesized that the CSF possesses a protein(s) that binds to and transports endocannabinoids/NAE in a manner analogous to intracellular FABP and serum albumin.

Lipocalin-type prostaglandin D synthase (L-PGDS; EC:5.3.99.2) is a secreted enzyme and is the second most abundant protein in human CSF (Hoffmann et al., 1993). L-PGDS converts prostaglandin H_2 to prostaglandin D_2, a metabolite that is also implicated in the regulation of sleep (Saper, Romanovsky, & Scammell, 2012; Zeitzer, 2013). In addition to its enzymatic function, L-PGDS binds to and serves as a carrier for hydrophobic ligands including retinoids, hemoglobin metabolites, thyroid hormones, gangliosides, and fatty acids (Kume et al., 2012; Mohri et al., 2006; Tanaka et al., 1997; Zhou et al., 2010). The large and unusually shaped binding cavity of L-PGDS confers an ability to bind a broad range of ligands, and upon ligand binding, the protein undergoes conformational changes to become more compact, aiding in higher-affinity binding (Inoue, Yagi, Urade, & Inui, 2009; Kumasaka et al., 2009; Shimamoto et al., 2007). L-PGDS belongs to the lipocalin family of proteins, which consist of a highly conserved fold that is characterized by orthogonal β-sheets that form the lipid ligand binding pocket (Flower, North, & Sansom, 2000). Interestingly, L-PGDS is the only known lipocalin that acts as both an enzyme and a lipid transporter (Hoffmann et al., 1993). Despite sharing low amino-acid sequence homology, FABP and lipocalins, including L-PGDS, share a similar overall structural fold and likewise bind to a diverse array of hydrophobic ligands (Elmes et al., 2015; Flower et al., 2000; Furuhashi & Hotamisligil, 2008). Given the role of endocannabinoids and related NAE in numerous physiological processes, their presence in CSF, and the structural similarity and ligand-

binding promiscuity between FABP and L-PGDS, we hypothesized that L-PGDS may serve as an endocannabinoid/NAE binding protein in the CSF.

Materials and Methods

Protein Purification

Residues 29–190 of L-PGDS were amplified from a human brain cDNA library and cloned into a pTXB1 vector (the first 22 *N*-terminal residues of L-PGDS comprise a hydro-phobic signal sequence that is posttranslationally cleaved). The expression construct was transformed into BL21(DE3) *Escherichia coli*, and recombinant [1–28] L-PGDS was purified using the IMPACT purification system (New England Biolabs, Ipswich, UK) as described previously (Kaczocha, Vivieca, Sun, Glaser, & Deutsch, 2012). Residual endogenous bacterial lipids were removed by incubation in a column of hydroxypropyl-beaded dextran for 1 h at 37 °C. The final delipidated protein was concentrated to 10 mg/mL in phosphate buffered saline (PBS) + 150 mM NaCl and flash-frozen with liquid nitrogen.

Fluorescence Displacement Binding Assays

Fluorescent binding assays were performed in 96-well Cost-ar® assay plates (Corning Life Sciences, Kennebunk, ME, USA). 12-*N*-methyl-(7-nitrobenz-2-oxa-1,3-diazo)aminostearic acid (NBD)-stearate was purchased from Avanti Polar Lipids (Alabaster, AL, USA). 11-(dansylamino)undecanoic acid (DAUDA) and 1-anilinonaphthalene-8-sulfonic acid (ANS) were purchased from Cayman Chemical Company (Ann Arbor, MI, USA).

Purified L-PGDS (3 μM) was incubated with DAUDA (500 nM) or ANS (500 nM) in 30 mM Tris–HCl and 100 mM NaCl buffer (pH 7.6). Competitor test compounds (0.1–250 μM) were then added to the wells, mixed, and the system was allowed to reach equilibrium by incubating in the dark at 25 °C for 20 min. All experimental conditions were tested in triplicate. Each independent assay included wells containing a strong competitive binder (retinoic acid or oleic acid [OLA], 10 μM) as a positive control for probe displacement and background readings (absence of protein in wells). Loss of fluorescence intensity was monitored with an F5 Filtermax Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA, USA) using excitation (ex.) and emission (em.) wavelengths appropriate for each respective probe (DAUDA ex./em. = 345/535 nm, NBD-stearate ex./em. = 465/535 nm, ANS ex./em. = 370/465 nm). Following background subtraction, the fluorescence intensity values were normalized and fitted to a one-site binding analysis using the GraphPad Prism software (Prism version 7.0 for Mac OS; Graphpad Software Inc., La Jolla, CA, USA) to determine the K_i of the tested compounds from the equation $K_i = IC_{50}/(1 + ([DAUDA]/K_d))$.

Determination of Fluorescent Probe Binding Affinity

Recombinant L-PGDS (1 μM) was titrated with DAUDA (0–16 μM), ANS (0–25 μM), or NBD (0–25 μM). The raw fluorescence intensity at each data point was corrected by subtracting the signal from each respective probe concentration in the absence of protein. K_d and B_{max} were then calculated by fitting the titration curve to the single-site saturation binding equation $Y = [B_{max} \times X/(K_d + X)]$ using the GraphPad Prism software.

Intrinsic Tryptophan Fluorescence Quenching Assay

Compounds were dissolved in dimethyl sulfoxide (DMSO) to a stock concentration of 2 mM. Compound solutions (10 μL) were added to 990 μL 5 mM Tris–HCl (pH 8.0) containing [1–28] L-PGDS (1.5 μM). The system was allowed to equilibrate for 30 min at 25 °C, and the intrinsic tryptophan fluorescence of the protein was measured (ex./em. 282/340 nm; 5 nm slit width) using a FP-6200 spectrofluorimeter (JASCO, Tokyo, Japan). Effects on tryptophan fluorescence resulting from nonspecific interactions with each compound were corrected with N-acetyl-L-tryptophanamide (1.5 μM).

Statistics

All quantitative data are expressed as means ± standard error (*SE*) from at least three independent experiments.

Results and Discussion

Human [1–28] L-PGDS was purified from *E. coli*, delipi-dated, and its purity confirmed by Coomassie staining (Fig. 1a). We employed fluorophore displacement assays to assess the binding affinities of ligands to [1–28] L-PGDS (Kume et al., 2012). [1–28] L-PGDS bound to the fluores-cent probe DAUDA with an affinity of 0.96 ± 0.08 μM (Fig. 1b), while binding to ANS was weaker ($K_d = 3.4 \pm 0.8$ μM), and NBD-stearate did not exhibit appreciable binding. These probe affinities to L-PGDS are generally in agreement with the values found in the existing literature (Breustedt, Schonfeld, & Skerra, 2006). Consequently, we employed DAUDA for all subsequent binding studies unless otherwise stated.

We examined the binding affinities of several classes of endogenous and synthetic ligands to L-PGDS (Fig. 2). The fatty acids OLA and palmitoleic acid (PLA) were selected because these fatty acids were previously shown to interact with L-PGDS (Kume et al., 2012; Zhou et al., 2010). We examined the binding of AEA, 2-AG, and their respective cyclooxygenase metabolites prostamide $F_{2\alpha}$ and prostaglandin E_2-glyceryl ester (PGE$_2$-GE), which were compared to prostaglandin $F_{2\alpha}$ (PGF$_{2\alpha}$) and prostaglandin E_2 (PGE$_2$). The cyclooxygenase metabolites were selected because of their involvement in pain and inflammation (Gatta et al., 2012; Hu, Bradshaw, Chen, Tan, & Walker, 2008). The NAE palmitoylethanolamide (PEA) and OEA, which activate the nuclear peroxisome proliferator-activated receptor alpha, and the primary fatty acid amide oleamide were selected because of their biological relevance and presence in the CSF. Furthermore, we examined the binding of Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD), the most abundant cannabinoids found in marijuana, which were recently shown to bind the brain-expressed FABP (Elmes et al., 2015). The binding of major THC metabolites and SBFI-26—a recently developed FABP inhibitor—were also explored (Berger et al., 2012).

OLA and PLA bound [1–28] L-PGDS with K_i values of 1.8 ± 0.9 and 3.7 ± 0.5 μM, respectively (Fig. 3f and Table 1). In contrast, AEA and 2-AG did not bind to [1–28] L-PGDS as evidenced by a lack of DAUDA displacement (Table 1). To rule out the possibility that the inability of endocannabinoids to displace DAUDA from L-PGDS may have been inherent to the probe, we repeated the experiments using ANS and similarly observed a lack of affinity

of AEA for [1–28] L-PGDS. PGF$_{2\alpha}$ weakly bound [1–28] L-PGDS (K_i = 81 ± 9 μM); however, prostamide F$_{2\alpha}$, the cyclooxygenase metabolite of AEA, was not found to bind with any appreciable affinity (Fig. 3c and Table 1). In contrast, PGE$_2$-GE, the cyclooxygenase metabolite of 2-AG, demonstrated a moderate affinity for [1–28] L-PGDS (K_i = 11.2 ± 1.2 μM) (Fig. 3f and Table 1). Interestingly, the cyclooxygenase metabolite of arachidonic acid, PGE$_2$, displayed much weaker binding (K_i = 128 ± 16 μM) than PGE$_2$-GE (Fig. 3d and Table 1). PEA and OEA did not display any appreciable binding to [1–28] L-PGDS.

The phytocannabinoids THC and CBD both weakly interacted with [1–28] L-PGDS (K_i = 175 ± 26 and 77.9 ± 3.9 μM, respectively) (Fig. 3a, b). Following con-sumption of marijuana, the cytochrome P450 system hydroxylates THC to its primary metabolite 11-hydroxy-[9]-THC (11-OH-THC), which in turn is further oxidized to the secondary metabolite 11-nor-9-carboxy-[9]-THC (THC-COOH) (Wall & Perez-Reyes, 1981). Intriguingly, 11-OH-THC and THC-COOH exhibited much higher affinities for L-PGDS than the parent compound (K_i = 61 ± 14 and 7.8 ± 1.7 M, respectively) (Fig. 3b). Similarly, ajulemic acid (AJA), a nonpsychoactive synthetic derivative of THC-COOH used to treat inflammatory pain, displayed strong affinity for [1–28] L-PGDS (K_i = 2.2 0.3 μM) (Fig. 3b) (Mitchell, Aslan, Safaei, & Vaughan, 2005). The FABP inhibitor SBFI-26 bound to L-PGDS with high affinity, similar to the fatty acids (K_i = 3.0 ± 0.5 μM) (Fig. 3e and Table 1).

Intrinsic tryptophan fluorescence quenching assays were employed as a secondary means of assessing relative *in vitro* affinities. Each compound was screened at 20 μM, and changes in intrinsic [1–28] L-PGDS fluorescence was monitored relative to the vehicle-treated protein (Table 1). As expected, all-trans retinoic acid, a known high-affinity L-PGDS ligand (K_d = 290 [notdef] 30 nM), displayed potent fluorescence quenching in this assay (71.1 ± 1.4%) (Breustedt et al., 2006). These data support and validate the results obtained from displacement assays, with lower K_i affinity values generally being predictive of higher quenching, and all compounds that were unable to displace DAUDA exhibited little to no (<4%) quenching.

This study was the first to thoroughly examine the binding of a variety of endogenous bioactive lipids, phytocannabinoids, and synthetic ligands to L-PGDS. [1–28] L-PGDS displayed the highest affinity for lipids bearing free carboxylate moieties while low or no affinity for ligands lacking this functional group, consistent with structural data demonstrating electrostatic interactions between the carboxylate groups of ligands and residues lining the binding cavity of L-PGDS (Lim et al., 2013). Contrary to our hypothesis, [1–28] L-PGDS displayed weak or no affinity for a variety of endogenous ligands including the endocannabinoids and NAE, suggesting that other proteins in the CSF may bind and transport these lipids. Indeed, serum albumin is present in CSF, and recent work indicates that apolipoprotein D binds to AEA (Huhmer, Biringer, Amato, Fonteh, & Harrington, 2006; Ruiz et al., 2013), potentially suggesting that these proteins facilitate CSF endocannabinoid transport. However, it is noteworthy that the levels of endocannabinoids in the CSF are orders of magnitude lower than those found in serum (Azim et al., 2015; Jumpertz, Guijarro, Pratley, Piomelli, & Krakoff, 2011), raising the possibility that these lipids may be present at sufficiently low concentrations to permit sufficient solubility in CSF. Additionally,

endogenous L-PGDS is highly glycosylated and may potentially display an altered ligand-binding profile than our data suggests due to inherent limitations of the bacterially expressed and truncated recombinant protein (Hoffmann, Nimtz, Wurster, & Conradt, 1994). Our study expands the repertoire of ligands that bind to L-PGDS and further indicates that this protein may serve as a carrier for a broad range of lipids in CSF; however, further investigations will be needed to validate any physiological implications from this work.

Acknowledgements

Abbreviations

2-AG	2-arachidonoylglycerol
11-OH-THC	11-hydroxy-Δ^9-tetrahydrocannabinol
AEA	*N*-arachidonoylethanolamine/anandamide
AJA	ajulemic acid
ANS	1-anilinonaphthalene-8-sulfonic acid
CBD	cannabidiol
CSF	cerebrospinal fluid
DAUDA	11-(dansylamino)undecanoic acid
FABP	fatty acid binding protein
L-PGDS	lipocalin-type prostaglandin D synthase
NAE	*N*-acylethanolamine
NBD	12-*N*-methyl-(7-nitrobenz-2-oxa-1,3-diazo) aminostearic acid
OEA	oleoylethanolamide
OLA	oleic acid
PEA	palmitoylethanolamide
PGE$_2$	prostaglandin E$_2$
PGE$_2$-GE	prostaglandin E$_2$-glyceryl ester
PGF$_{2\alpha}$	prostaglandin F$_{2\alpha}$
PLA	palmitoleic acid
THC	Δ^9-tetrahydrocannabinol
THC-COOH	11-nor-9-carboxy-Δ^9-tetrahydrocannabinol

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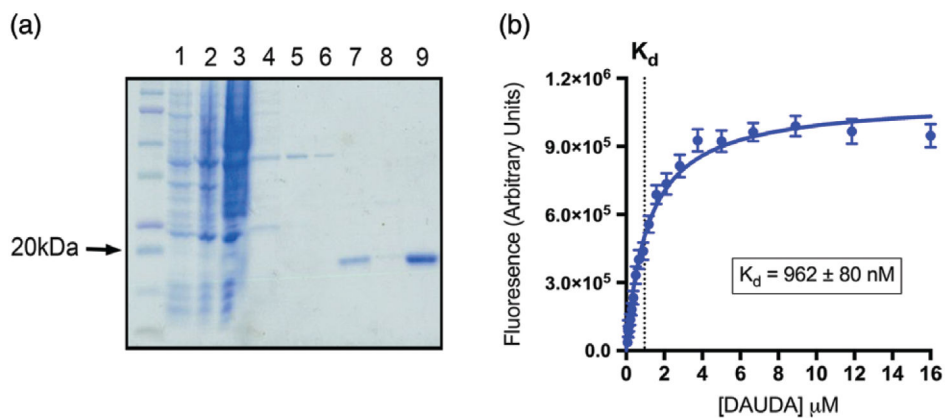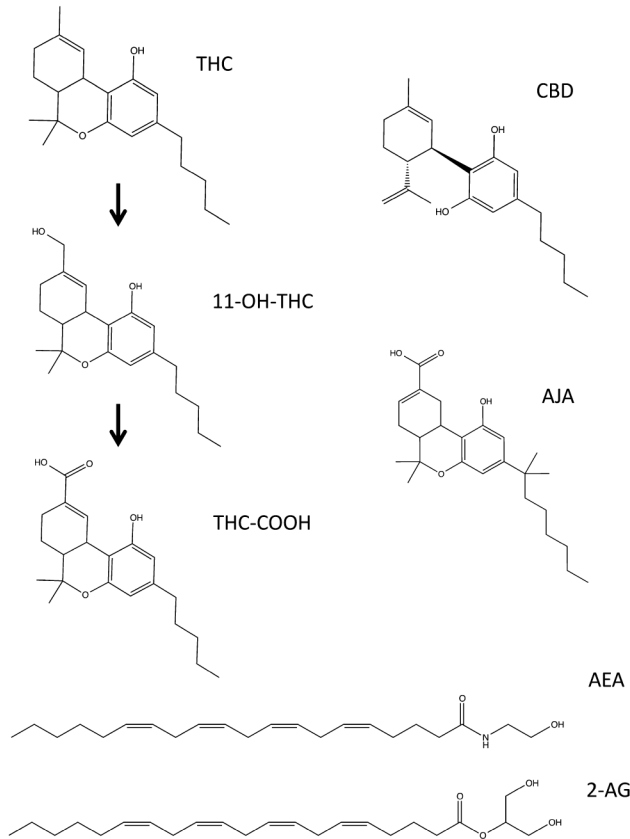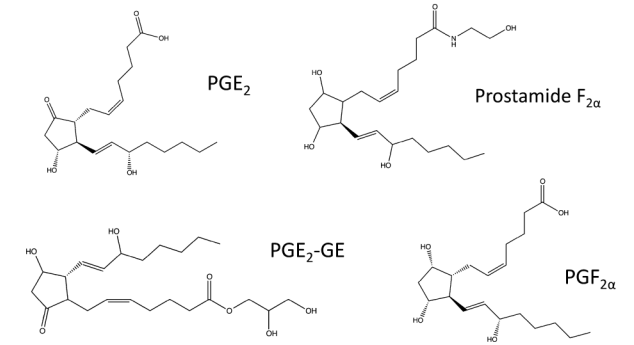
Fig. 1.
Lipocalin-type prostaglandin D synthase (L-PGDS) protein purification and determination of 11-(dansylamino)undecanoic acid (DAUDA) binding affinity. (a) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of [1–28] L-PGDS protein purification. Unlabeled lane is protein ladder; lane 1, uninduced bacterial cell lysate; lane 2, isopropyl β-D-1-thiogalactopyranoside (IPTG)-induced bacterial cell lysate; lane 3, column effluent (flow through); lane 4, first wash; lane 5, second wash; lane 6, third wash; lane 7, pooled elution fractions; lane 8, fast protein liquid chromatography (FPLC)-purified and delipidated protein; lane 9, concentrated final product. (b) Saturation curve of DAUDA binding to [1–28] L-PGDS

Cannabinoids

THC

11-OH-THC

THC-COOH

CBD

AJA

AEA

2-AG

Prostaglandins

PGE$_2$

Prostamide F$_{2\alpha}$

PGE$_2$-GE

PGF$_{2\alpha}$
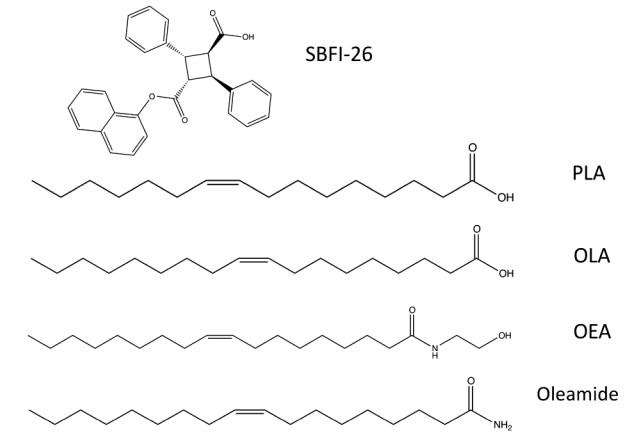
Other

SBFI-26

PLA

OLA

OEA

Oleamide

Fig. 2.
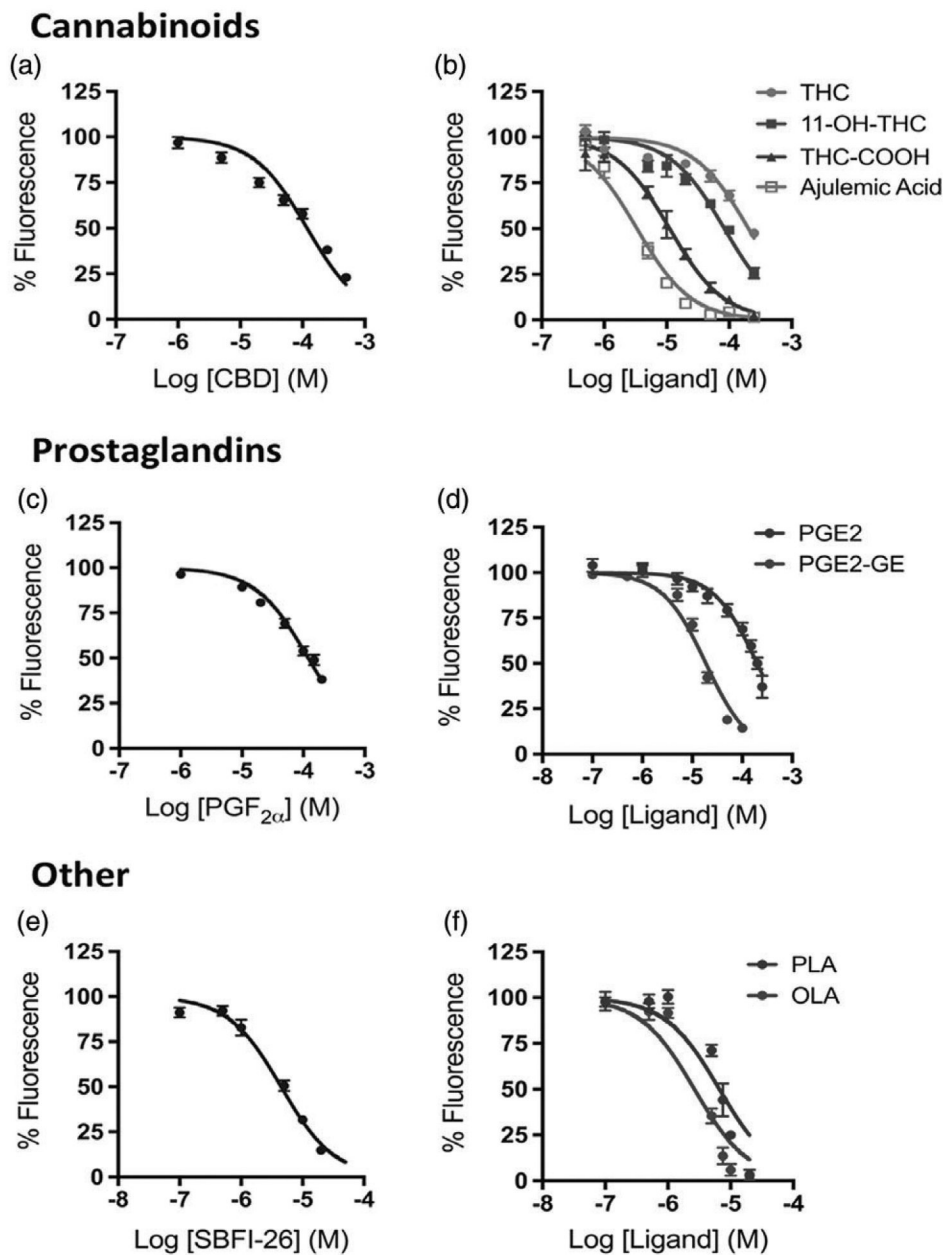Chemical structures of the tested compounds

Fig. 3.
Binding curves for active compounds. Displacement of 11-(dansylamino)undecanoic acid (DAUDA) from [1–28] lipocalin-type prostaglandin D synthase (L-PGDS) by (a) cannabidiol (CBD), (b) [9]-tetrahydrocannabinol (THC), THC metabolites (11-OH-THC and THC-COOH), and ajulemic acid (AJA), (c) prostaglandin F2α ($PGF_{2\alpha}$), (d) prostaglandin E_2 (PGE_2) and prostaglandin E_2-glyceryl ester (PGE_2-GE), (e) SBFI-26, and (f) fatty acids oleic acid (OLA) and palmitoleic acid (PLA). Displacement curve data presented as a mean $\pm$ *SE* from at least three independent assays. Solid lines represent optimal curve-fitting to a one-site competition model

Table 1

In vitro affinities of select compounds for [1–28] lipocalin-type prostaglandin D synthase (L-PGDS) as determined by fluorescence displacement binding assay and intrinsic tryptophan fluorescence quenching assay

Chemical class	Compound	K_i (μM)[a]	Quenching (%)[a,b]
Prostaglandins	Prostaglandin $F_{2\alpha}$ (PGF$_{2\alpha}$)	81 ± 9	2.7 ± 1.7
	Prostamide $F_{2\alpha}$	>200	0.1 ± 2.6
	Prostaglandin E_2 (PGE$_2$)	128 ± 16	6.5 ± 1.5
	Prostaglandin E_2-glyceryl ester (PGE$_2$-GE)	11.2 ± 1.2	15.1 ± 1.7
Phytocannabinoids	Cannabidiol (CBD)	77.9 ± 3.9	7.2 ± 2.1
	^{9}-Tetrahydrocannabinol (THC)	175 ± 26	14.0 ± 2.2
THC metabolites	11-Hydroxy-^{9}-tetrahydrocannabinol (11-OH-THC)	61 ± 14	9.7 ± 1.3
	11-Nor-9-carboxy-^{9}-tetrahydrocannabinol (THC-COOH)	7.8 ± 1.7	15.6 ± 1.3
Synthetic THC-COOH analog	Ajulemic acid (AJA)	2.3 ± 0.2	21.9 ± 2.9
Endocannabinoids	*N*-Arachidonoylethanolamine/anandamide (AEA)	>200	3.1 ± 3.3
	2-Arachidonoylglycerol (2-AG)	>200	-0.8 ± 3.0
NAE	Palmitoylethanolamide (PEA)	>200	1.3 ± 4.7
	Oleoylethanolamide (OEA)	>200	-0.9 ± 2.1
Fatty acid amide	Oleamide	>200	3.6 ± 2.0
Fatty acids	Oleic acid (OLA)	1.8 ± 0.9	15.1 ± 3.7
	Palmitoleic acid (PLA)	3.7 ± 0.5	11.9 ± 2.3
Synthetic FABP inhibitor	SBFI-26	3.0 ± 0.5	33.3 ± 2.2

[a]Values represent mean ± *SE* from at least three independent assays.

[b]Data expressed as % fluorescence reduction relative to vehicle-treated [1–28] L-PGDS.