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Cannabidiol-2',6'-Dimethyl Ether, a Cannabidiol Derivative, Is a Highly Potent and Selective 15-Lipoxygenase Inhibitor

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Running title page

a) **Running title:** Selective inhibition of 15-LOX by CBD-2',6'-dimethyl ether (52 characters)

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d) **ABBREVIATIONS:** CBD, cannabidiol; LOX, lipoxygenase; NDGA, nordihydroguaiaretic acid; LDL, low density lipoprotein; CBDM, CBD-2'-mono methyl ether; CBDD, CBD-2',6'-dimehyl ether.

ABSTRACT:

The inhibitory effect of nordihydroguaiaretic acid (NDGA), a non-selective lipoxygenase (LOX) inhibitor, -mediated 15-LOX inhibition has been reported to be affected by modification of its catechol ring such as methylation of the hydroxyl group. Cannabidiol (CBD), one of the major components of marijuana, is known to inhibit LOX activity. Based on the phenomenon observed in NDGA, we investigated whether or not methylation of CBD affects its inhibitory potential against 15-LOX, since CBD contains a resorcinol ring, which is an isomer of catechol. Although CBD inhibited 15-LOX activity with an IC_{50} value (50% inhibition concentration) of 2.56 μM, its mono-methylated and di-methylated derivatives, CBD-2'-monomethyl ether (CBDM) and CBD-2',6'-dimethyl ether (CBDD) inhibited 15-LOX activity more strongly than CBD. The number of methyl groups in the resorcinol moiety of CBD (as a prototype) appears to be a key determinant for potency and selectivity in inhibition of 15-LOX. The IC_{50} value of 15-LOX inhibition by CBDD is 0.28 μM, and the inhibition selectivity for 15-LOX (i.e., the 5-LOX/15-LOX ratio of IC_{50} values) is more than 700. Among LOX isoforms, 15-LOX is known to be able to oxygenate cholesterol esters in the low density lipoprotein (LDL) particle (i.e., the formation of oxidized LDL). Thus, 15-LOX is suggested to be involved in developing atherosclerosis, and CBDD may be a useful prototype for producing medicines for atherosclerosis.

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Introduction

Cannabidiol (CBD) is known to be one of the major components in the cannabis plant (Mechoulam, 1970; Turner et al., 1980; Dewey, 1986; Howlett et al., 2002). It has been reported that CBD can inhibit lipoxygenase (LOX) activity with relatively high concentrations (i.e., μM order) when compared with other plant-derived inhibitors, such as luteolin, nordihydroguaiaretic acid (NDGA), and quercetin (i.e., from nM to μM order) (Evans et al., 1987; Yamamoto et al., 1998; Whitman et al., 2002; Sadik et al., 2003; Russo, 2004). In addition to the LOX inhibitors mentioned above, there are many LOX inhibitors that have been discovered in plants. LOXs are non-heme iron-containing enzymes that catalyze the dioxygenation of polyunsaturated fatty acids, such as arachidonic acid and linolenic acids. Until now, three major LOX isoforms have been discovered (i.e., 5-, 12-, and 15-LOX) (Funk, 1996; Brash, 1999; Kuhn and Thiele, 1999). Among LOX isoforms, 15-LOX is known to be able to directly oxygenate not only free fatty acids but also complex substrates such as phospholipids, cholesterol ester, and the cholesterol ester in the low density lipoprotein (LDL) particle (Brash, 1999; Kuhn and Thiele, 1999; Takahashi and Yoshimoto, 2002). Oxidation of LDL is recognized as the first step for the development of atherosclerosis (Kühn et al., 1997), and the role of 15-LOX in the process of LDL oxidation and the progress of atherosclerosis have been extensively investigated using 15-LOX-knockout mice (Cyrus et al., 1999). However, the role of 15-LOX in initiating LDL

oxidation is controversial, because Sparrow and Olszewski have attributed the ability of 15-LOX inhibitors to block LDL modification to nonspecific antioxidant effects rather than direct effects on the enzyme (Sparrow and Olszewski, 1992). Thus, 15-LOX isoform-selective inhibitor is required to clarify the involvement of 15-LOX in the formation of oxidized LDL.

However, most of the plant-derived LOX inhibitors containing catechol and resorcinol rings inhibit LOX isoforms in a non-selective manner due to their antioxidant properties, resulting in the change of co-factor Fe(III) into Fe(II) (Sadik et al., 2003). Thus, there is a need to develop highly potent and LOX isoform-selective inhibitors. It has been reported that 15-LOX activity is quite sensitive to the structural modifications of NDGA's catechol ring, an isomer of resorcinol (Fig. 1A), such as methylation of its hydroxyl group (Blecha et al., 2007), although NDGA is well-known as a pan-LOX inhibitor (Hope et al., 1983; Whitman et al., 2002). There have been no reports investigating whether any cannabinoid(s) including CBD "selectively and potently" inhibit the 15-LOX isoform, while CBD having a resorcinol ring has been shown to inhibit LOX activity (Evans et al., 1987; Russo, 2004).

Based on the possibility of CBD as a prototype for 15-LOX inhibitor, 2'-mono methylated CBD (CBDM, Fig. 1A) and 2',6'-dimethylated CBD (CBDD, Fig 5A), whose respective phenolic hydroxyl groups of resorcinol ring are methylated, were synthesized from CBD. It was observed that unlike CBD, these methylated derivatives are highly potent and selective inhibitors for 15-LOX, especially

CBDD lacking free phenolic hydroxyl groups (the IC_{50} = 280 nM), without inhibitory effect on 5-LOX. The potential utility of CBDD as a probe for analyzing the formation of oxidized LDL is discussed.

Materials and Methods

Cannabinoids and Chemicals. Δ^9-Tetrahydrocannabinol (THC), cannabinol (CBN), CBD, and cannabidiolic acid (CBDA) were isolated and purified from the cannabis leaves according to the methods described elsewhere (Aaramaki et al., 1968). CBDM, CBDD, cannabielsoin (CBE), and CBE monomethyl ether (CBEM) were prepared as described previously (Gohda et al., 1990). CBD-hydroxyquinone (CBDHQ) was synthesized according to the method by Mechoulam et al. (Mechoulam et al., 1968). Purities of these cannabinoids were checked to be at least above 95% by gas chromatography (Watanabe et al., 2005; Takeda et al., 2008). NDGA was purchased from Cayman Chemical Company (Ann Arbor, MI). All other reagents were of analytical grade.

Enzyme Sources. Measurements of the 5-LOX and 15-LOX activities were carried out using a commercially available LOX inhibitor screening assay kit (Cayman Chemical Company, Ann Arbor, MI). 5-LOX (lot nos. 0400028-1) and 15-LOX (lot nos. 193367-193368) screening enzymes were purchased from Cayman Chemical Company (Ann Arbor, MI). All inhibitors added to the reaction system were prepared just before use. After enzyme reactions, resulting hydroperoxides were treated with chromogen to develop the reaction and then

absorbance intensities were determined spectrophotometrically with a 96-well plate reader at 490 nm. No colorimetric change was observed in control incubations that were performed by omitting enzymes or with heat denatured enzymes and inhibitors in combination with chromogen. The concentrations of cannabinoids used in this study were determined based on the solubility and the concentration without interference with chromogen. Each assay was performed in triplicate.

Data Analysis. The concentration of the inhibitor that is required to produce 50% inhibition of the enzymatic activity (IC_{50}) was determined from the curves plotting enzymatic activity versus inhibitor concentrations using Origin7.5J software (OriginLab Corp., Northampton, MA). The details of the calculations were described in our previous articles (Takeda et al., 2006; Takeda et al., 2008). Differences were considered to be significant when the *p* value was calculated to be less than 0.05. All statistical analyses were performed by Scheffé's F test, which is a type of post-hoc test for analyzing results of ANOVA testing. These calculations were done using Statview5.0J software (SAS Institute Inc., Cary, NC).

Results

Effects of Cannabidiol and Its Derivative on LOX Activity. In the present study, we first synthesized a derivative of CBD, CBDM (Fig. 1A) because it was reported that the inhibitory effect of NDGA on 15-LOX activity was shown to be

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modified by methylation of the hydroxyl group of its catechol ring (Blecha et al., 2007). The inhibitory effects of CBD, CBDM, and NDGA on 5/15-LOX-catalyzed oxygenation activity were examined using purified LOX as enzyme sources. Although 5-LOX activity was not significantly inhibited by the addition of 2 μM of CBD and CBDM except for NDGA, 15-LOX activity was quite sensitive to these inhibitors investigated, especially CBDM (complete inhibition) and NDGA (94% inhibition) compared with control activity (100%) (Fig. 1B; see also structures in Fig. 1A). It is important to know the inhibition selectivity (5-LOX/15-LOX ratio of IC_{50} values) of CBD and CBDM-mediated inhibition of LOX isoforms. Thus, we next determined IC_{50} values for the inhibition of the two LOX isoforms by CBD and CBDM. It was shown that CBD had the potential to inhibit both 5 and 15-LOX isoforms, but 15-LOX activity was more selectively inhibited by CBD than 5-LOX, which gives rise to the ratio of 28.8 (Fig. 2A and 2B, Table 1). On the other hand, compared with CBD-produced inhibition of 15-LOX enzyme, CBDM exhibited much greater inhibitory effect on 15-LOX activity than CBD; namely, 1) the IC_{50} value of 15-LOX inhibition is 720 nM, which is around 3.6-times stronger than CBD and 2) 5-LOX activity was not remarkably inhibited even in the presence of 200 μM CBDM (around 75%), although 5-LOX was inhibited by CBD with the IC_{50} value of 73.73 μM. Thus, the inhibition selectivity of CBDM is determined to be at least 278, which is around 9.6-times greater than that of CBD (Fig. 2A and 2B, Table 1). In short, it was revealed that CBDM, which has a methylated 2'-hydroxyl group, is much more effective/selective in

inhibition of 15-LOX than CBD. Based on the results obtained in Fig. 2, the following experiments focused on the inhibition of 15-LOX activity by "methylated" CBD.

Structural Requirement for Inhibitory Effect of CBD and Its Derivatives on 15-LOX Activity. It is well known that the inhibitory potential for LOXs by flavonoids depends on the details of their structures (the number of aromatic rings and hydroxyl groups, planarity, etc.). Here, we performed experiments to obtain information about structure-inhibition relationships by focusing on the structures of CBD and its structurally related cannabinoids (i.e., CBDA, CBE, CBEM, CBN, and THC) (Fig. 3A). 15-LOX activities were inhibited by 10 μM CBN, 2 μM CBD, and 0.7 μM CBDM to the same extent (~50%) when compared with controls (Fig. 3B). The concentrations of CBD and CBDM were determined based on their IC_{50} values for 15-LOX inhibition (see Fig. 2, Table 1). The degree of inhibition by THC was much stronger than that of CBN, although THC was a weak inhibitor for 15-LOX when compared with CBDM (Figs. 2A and 3B), indicating that the free 6'-substitution group, not available for the ring formation with the terpene moiety, is important for the inhibition, and the number of aromatic rings is also important (Figs. 1A and 3A). CBDA, an acid form of CBD, having a carboxyl group at its 3'-position (Fig. 3A), did not have any inhibitory effect on the activity (Fig. 3C). In agreement with this result, it has been reported that 2,4-dihydroxy-benzoic acid does not fit into the active site of 15-LOX (Borbulevych et al., 2004). Furthermore, CBE and CBEM, whose 2'-hydroxyl

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groups are used in ring formation with neighbor structures, did not exert any inhibitory effects on 15-LOX activity (Fig. 3). Taken together, it is suggested that the core resorcinol itself not being substituted, which is also freed from ring formation with neighbor structure (i.e., planar structure is unfavorable), is a key determinant for the inhibition.

15-LOX Activity Is Not Sensitive to a Hydroxy-quinone Form of CBD. This experiment was performed to investigate whether or not CBD hydroxy-quinone (CBDHQ, Fig. 4A), an oxidized product of CBD (Watanabe et al., 1991; Usami et al., 2008), is a possible active form of CBD-mediated 15-LOX inhibition because it has been suggested that LOX enzymes have a co-oxidase activity as well as an oxygenase activity (Kulkarni, 2001). Furthermore, the formation of oxygenated products by 15-LOX activity has been reported to be inhibited by AA-861, a benzoquinone derivative (Li et al., 2004), although AA-861 exhibits preference for the inhibition of 5-LOX (Ashida et al., 1983). However, 15-LOX activity was not inhibited by CBDHQ, whereas the same concentration of CBD inhibited 15-LOX activity (13%) (Fig. 4B, see also Fig. 2A). Thus, it is suggested

that CBD may exert its inhibitory effect on 15-LOX as CBD itself but not as the quinone form.

Effect of Dimethylated-Form of CBD on 5/15-LOX Activity. Evidence from the present study so far suggests that methylation of the hydroxyl group of resorcinol in CBD (i.e., CBDM) was found to be effective in inhibition of 15-LOX

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with reduced inhibitory potential toward 5-LOX. However, 5-LOX activity was inhibited by CBDM in a concentration-dependent manner (Fig. 2B). We hypothesized that if methylation of the hydroxyl group at the 2'-position on CBD is effective for 15-LOX inhibition, further methylation of the remaining free hydroxyl group at the 6'-position in CBDM into methoxyl group (i.e., CBDD) might strengthen the degree of CBDM-mediated inhibition of 15-LOX. Thus, we next synthesized CBDD (Fig. 5A), and it was subjected to an inhibition assay against 5/15-LOX. In support of our expectation, it was revealed that CBDD was the most effective inhibitor for 15-LOX without inhibition of 5-LOX compared with CBD and CBDM (Figs. 2 and 4B, Table 1). The IC_{50} value for 15-LOX inhibition is 0.28 μM, which is around 2.6-fold and 9.1-fold stronger potential than CBDM and CBD, respectively. The 5-LOX/15-LOX ratio of IC_{50} values is determined to be at least 700 (Fig. 5B and Table 1). Taken together with the results obtained in Fig. 2, it is clearly demonstrated that the methylation of resorcinol ring in CBD is highly effective in 15-LOX selective inhibition.

Discussion

In the current study, it was revealed that CBD and its methylated forms, CBDM and CBDD, have a selective inhibitory effect on 15-LOX-catalyzed oxygenation. In particular, CBDD was shown to be a highly potent and selective inhibitor for 15-LOX (Fig. 5B, Table 1). It has been reported that quercetin is able to inhibit 15-LOX activity by competitive mechanisms, although it contains

resorcinol and catechol, having antioxidant activity in the structure (Borbulevych et al., 2004). To explain this phenomenon, it has been reported that quercetin is subjected to oxidation via co-oxidase activity of LOX during oxygenation reaction. Thus, we first focused on the metabolites of CBD and CBDM as possible ultimate forms for 15-LOX inhibition because they can be metabolized into CBE and CBEM, respectively, by oxidation (Fig. 3A) (Gohda et al., 1990; Yamamoto et al., 1991). However, 15-LOX activity was not inhibited even in high concentration of CBE (10 μM) and also CBEM (10 μM) (Fig. 3B), suggesting that the cannabielsoin-type metabolites are not involved in inhibition by CBD and CBDM. CBD can also be metabolized into CBDHQ by oxidation such as cytochrome P450 (P450)-catalyzed reaction (Bornheim and Grillo, 1998). As mentioned above, LOX has co-oxidase activity as well. We next investigated whether or not CBDHQ, an oxidized product of CBD, can affect 15-LOX activity. However, 15-LOX was not inhibited by CBDHQ (Fig. 4B). No inhibition by CBDHQ was also observed in case of 5-LOX (data not shown). We previously reported that no demethylated products were observed when CBDM and CBDD were reacted with guinea pig liver microsomes that contain many drug-metabolizing enzymes including P450 (Gohda et al., 1990; Yamamoto et al., 1991; Oguri et al., 1994; Ishii et al., 2005; Takeda et al., 2005a; Takeda et al., 2005b; Takeda et al., 2009), although CBDM can be converted into CBEM in microsomes (Gohda et al., 1990; Yamamoto et al., 1991). Thus, it is thought that the inhibition potential of CBD and CBDM might be weaken in near *in vivo*

situations, although in the absence of drug-metabolizing enzymes, CBD and CBDM behave as selective 15-LOX inhibitors (Fig. 2, Table 1). Taking into consideration these lines of evidence, it is suggested that 15-LOX inhibition by CBDM and CBDD, including CBD, might be caused by their structural nature themselves, although we could not exclude the possibility that CBD and CBDM-mediated inhibition of 15-LOX is also attributable to its antioxidant property (i.e., phenol and resorcinol moieties). LOX enzymes are mostly known for their peroxidase activity and metabolizing unsaturated fatty acids. However, until now, their co-oxidase activity is much less explored. Studies on molecular mechanism(s) underlying CBDD-mediated inhibition of 15-LOX are under investigation.

It has been reported that THC can suppress the progression of atherosclerosis via cannabinoid receptors in animal models, while CBDD has shown to have no affinity for the receptor (Thomas et al., 2004; Steffens and Mach, 2006). The extent of THC-mediated inhibition of 15-LOX is very weak when compared with that of CBDD (i.e., the IC_{50} values are 2.42 μM and 0.28 μM, respectively, Figs. 3B and 5B), suggesting that these cannabinoids might be able to abrogate atherosclerosis via different approaches.

There are pharmaceutical agents for the therapy of atherosclerosis, which can decrease LDL levels. However, the agents tend to lower total LDL but not only “oxidized LDL, and oxidized LDL can be produced by nonspecific free-radical oxidation (Sparrow and Olszewski, 1992). We propose the possibility that

CBDD might be a useful prototype for producing a specific 15-LOX inhibitor without containing a phenolic hydroxyl group in its structure different from NDGA, CBD, and CBDM (Figs. 1A and 5A). Furthermore, CBDD can be used as a probe in analyzing mechanism(s) of LDL oxidation by 15-LOX because of potent and 15-LOX isoform-selective inhibition.

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Footnotes

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Legends for figures

FIG. 1. Effects of CBD, CBDM, and NDGA on 5-LOX and 15-LOX activities. Structures of CBD, CBDM, and NDGA are shown in (A). (B), enzymatic reactions were initiated with linoleic acid, and then chromogen was added to stop the reactions and to develop colorimetric reactions. The absorbance was monitored at 490 nm. Details of the assay conditions are described under *Materials and Methods.* Each bar represents the mean ± S.D. (triplicate determinations) of the relative activity to the control. *significantly different ($p < 0.05$) from control. N.D., not detectable (due to complete inhibition). Figures in CBD and CBDM are numbered according to the monoterpenoid nomenclature.

FIG. 2. Dose-dependent inhibition by CBD and CBDM on LOX activity. (A) and (B) Lipoxygenation by two isoforms of LOX enzymes (A, 15-LOX: B, 5-LOX) was examined in the presence of indicated concentrations of CBD and CBDM. Reactions were initiated with linoleic acid, and then chromogen was added to stop the reactions and to develop colorimetric reactions. The absorbance was monitored at 490 nm. Details of the assay conditions are described under *Materials and Methods.* Each bar represents the mean ± S.D. (triplicate determinations) of the relative activity to the control.

FIG. 3. Effects of CBD and its structurally related cannabinoids on 15-LOX activity. (A), structures of cannabinoids tested (CBDA, CBE, CBEM, CBN, and THC). (B) and (C), effects of CBD and its structurally related cannabinoids on 15-LOX activity were examined in the presence of indicated concentrations of cannabinoids. Reactions were initiated with linoleic acid, and then chromogen was added to stop the reactions and to develop colorimetric reactions. The absorbance was monitored at 490 nm. Details of the assay conditions are described under *Materials and Methods.* Each bar represents the mean ± S.D. (triplicate determinations) of the relative activity to the control. *significantly different (p < 0.05) from control. Figures in cannabinoids are numbered according to the monoterpenoid nomenclature.

FIG. 4. Effect of CBDHQ on 15-LOX activity. 15-LOX activity was examined in the presence of 0.25 μM CBDHQ or CBD (the concentration was determined based on the inhibition of CBD for 15-LOX: see Fig. 2A). Reactions were initiated with linoleic acid, and then chromogen was added to stop the reactions and to develop colorimetric reactions. The absorbance was monitored at 490 nm. Details of the assay conditions are described under *Materials and Methods.* Each bar represents the mean ± S.D. (triplicate determinations) of the relative activity to the control. Interference was observed when CBDHQ was added with concentrations greater than 0.5 μM.

Fig. 5. Dose-dependent inhibition by CBDD on 15-LOX activity. (A), structure of CBDD. (B), effect of CBDD on 5-LOX and 15-LOX activity was examined in the presence of indicated concentrations of cannabinoids. Reactions were initiated with linoleic acid, and then chromogen was added to stop the reactions and to develop colorimetric reactions. Effects of CBD and CBDM on LOX activities were also investigated in this experiment, and similar results were obtained (see Fig. 2; data not shown). The absorbance was monitored at 490 nm. Details of the assay conditions are described under *Materials and Methods.* Each bar represents the mean ± S.D. (triplicate determinations) of the relative activity to the control.

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TABLE 1 Comparison of IC_{50} values (μM) of CBD and its methylated forms

Inhibitors	5-LOX	15-LOX-	5-LOX/15-LOX ratio[a]
CBD	73. 73	2.56	28.8
CBDM	200<	0.72	278
CBDD	200<	0.28	714

[a]The ratio of the IC_{50} values for 5-LOX and 15-LOX can be used as an indication of the 15-LOX selectivity of inhibitors. A 5-LOX/15-LOX ratio of more than 1 indicates preferential 15-LOX selectivity.

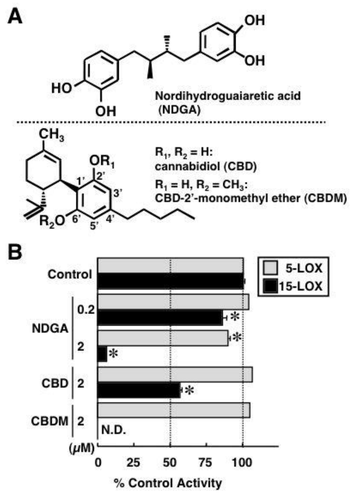

Fig. 1

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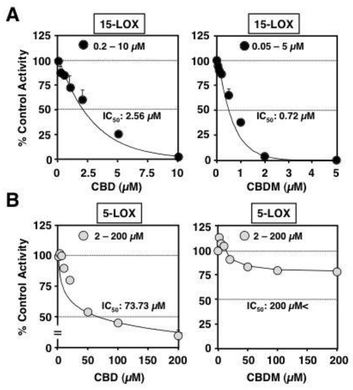

Fig. 2

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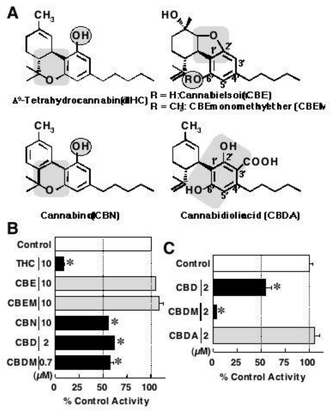


Fig. 3

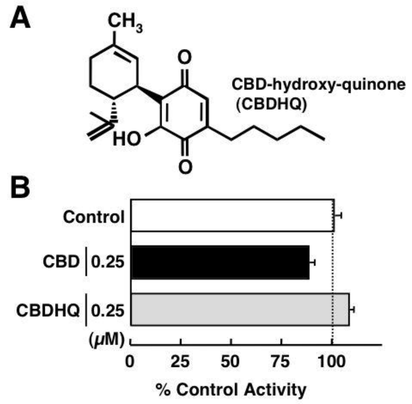
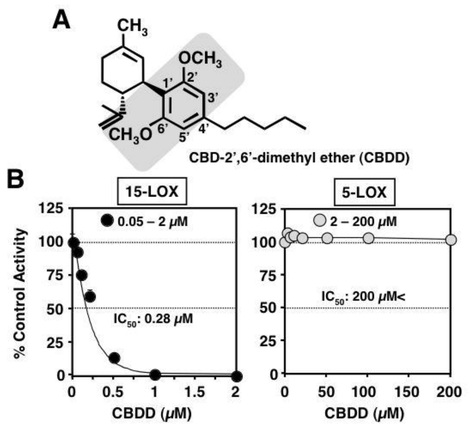


Fig. 4

Fig. 5



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