Characterization of the structural determinants required for potent mechanism-based inhibition of human cytochrome P450 1A1 by cannabidiol

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Abbreviations: CBD, cannabidiol; CBDHQ, CBD-hydroxyquinone; CBDM, CBD-2 -monomethyl ether; CBDD, CBD-2 ,6 -dimethyl ether; CBDV, cannabidivarin; CYP, cytochrome P450; EROD, 7-ethoxyresorufin *O*-deethylase.

Abstract

We previously demonstrated that cannabidiol (CBD) was a potent mechanism-based inhibitor of human cytochrome P450 1A1 (CYP1A1). However, the moiety of CBD that contributes to the potent mechanism-based inhibition of human CYP1A1 remains unknown. Thus, the effects of compounds structurally related to CBD on CYP1A1 activity were examined with recombinant human CYP1A1 in order to characterize the structural requirements for potent inactivation by CBD. When preincubated in the presence of NADPH for 20 min, olivetol, which corresponds to the pentylresorcinol moiety of CBD, enhanced the inhibition of the 7-ethoxyresorufin *O*-deethylase activity of CYP1A1. In contrast, *d*-limonene, which corresponds to the terpene moiety of CBD, failed to inhibit CYP1A1 activity in a metabolism-dependent manner. Pentylbenzene, which lacks two free phenolic hydroxyl groups, also did not enhance CYP1A1 inhibition. On the other hand, preincubation of the CBD-2 -monomethyl ether (CBDM) and CBD-2 ,6 -dimethyl ether (CBDD) enhanced the inhibition of CYP1A1 activity. Inhibition by cannabidivarin (CBDV), which possessed a propyl side chain, was strongly potentiated by its preincubation. Orcinol, which has a methyl group, augmented CYP1A1 inhibition, whereas its derivative without an alkyl side chain, resorcinol, did not exhibit any metabolism-dependent inhibition. The preincubation of CBD-hydroxyquinone did not markedly enhance CYP1A1 inhibition. We further confirmed that olivetol, CBDM, CBDD, CBDV, and orcinol, as well as CBD (k_{inact} = 0.215 min^{-1}), inactivated CYP1A1 activity; their k_{inact} values were 0.154, 0.0638, 0.0643, 0.226, and 0.0353 min^{-1}, respectively. These results suggest that the methylresorcinol structure in CBD may have structurally important roles in the inactivation of CYP1A1.

Keywords: cannabidiol; CYP1A1; inactivation; mechanism-based inhibition; structural requirement.

1. Introduction

Cannabidiol (CBD) is one of the major constituents in marijuana [1]. Although another one of the major phytocannabinoids, Δ^9-tetrahydrocannabinol, is psychoactive, CBD is not. It has been shown to exhibit several pharmacological effects such as prolonging drug-induced sleep as well as antiepileptic, anxiolytic, antiemetic, and anti-tumorigenic actions [2, 3]. Some of these effects may be of therapeutic importance. Sativex®, a medicine made from marijuana extracts containing CBD, has been clinically used for the symptomatic relief of neuropathic pain in patients with multiple sclerosis.

CBD is also known to inhibit CYP-mediated drug metabolism. The administration of CBD has been shown to reduce the systemic clearance of hexobarbital, which is metabolized by cytochrome P450 2C9 (CYP2C9), in humans [4]. We more recently demonstrated that CBD inhibited the catalytic activities of CYP1A1, CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP3A4, and CYP3A5 in human liver microsomes and/or recombinant CYP enzymes [5-10]. We also showed that CBD more potently inhibited the activity of human CYP1A1 than the other CYP enzymes examined so far [5]. Furthermore, CBD had competitive and mechanism-based inhibitory effects on human CYP1A1 [5].

CBD contains pentylresorcinol and terpene moieties and is rotatable between their moieties (Fig. 1). We previously elucidated the structural requirements for the inhibition of cyclooxygenase, lipoxygenase, and CYP by CBD and its derivatives [6-8, 10-12]. Our direct inhibition study with CBD derivatives and molecular modeling revealed that the pentylresorcinol structure in CBD had structurally important roles in direct CYP1A1

inhibition; however, the whole structure of CBD is known to be required for overall inhibition [13]. These studies on structure-inhibition relationships may provide an insight into the mechanism(s) underlying the potent inhibition of human CYP1A1 by CBD. However, the structural requirements for the potent mechanism-based inhibition of human CYP1A1 by CBD remain unclear.

In the present study, the inactivating effects of CBD-related compounds (Fig. 1) on human CYP1A1 activity were examined to elucidate the structural requirements for potent inactivation by CBD. We report herein that the methylresorcinol structure in CBD may play important roles in CYP1A1 inactivation.

2. Materials and methods

2.1. Materials

CBD was isolated from cannabis leaves using a previously reported method [14]. The CBD-2 -monomethyl ether (CBDM) and CBD-2 ,6 -dimethyl ether (CBDD) were prepared as described previously [15]. CBD-hydroxyquinone (CBDHQ) was synthesized according to the method of Mechoulam et al. [16]. The purities of these cannabinoids were determined to be above 97% by gas chromatography, except for CBDD, the purity of which was 93% [17]. Cannabidivarin (CBDV) was generously provided by Dr. Yukihiro Shoyama at Nagasaki International University (Sasebo, Japan). Other chemicals and materials were obtained from the following sources: microsomes from baculovirus-infected insect cells expressing CYP1A1 with NADPH-CYP reductase (Supersomes™) from BD Gentest (Woburn, MA);

7-ethoxyresorufin, resorufin, olivetol, *d*-limonene, and pentylbenzene from Sigma-Aldrich Corp. (St. Louis, MO); NADPH from Oriental Yeast Co. Ltd. (Tokyo, Japan); resorcinol from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); orcinol obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). All other chemicals and solvents used were of the highest quality commercially available.

2.2. Inactivation studies

Inhibition experiments were conducted using 96-well microtiter plates, as described below, to identify the potential metabolism-dependent inhibition of human CYP1A1 by CBD-related compounds. The preincubation mixture consisted of recombinant CYP1A1 (10 fmol), each CBD-related compound (up to 100 μM), 1.67 mM NADPH, and 50 mM Tris-HCl buffer (pH 7.4) containing 1% fatty acid-free bovine serum albumin in a final volume of 180 μl. After pre-warming at 37°C for 5 min, the preincubation was initiated by the addition of NADPH. 7-ethoxyresorufin *O*-deethylase (EROD) reactions were initiated following a 20-min preincubation by adding 20 μl of 7-ethoxyresorufin solution to the preincubation mixture (final substrate concentration, 150 nM), as described previously [5]. Fluorescence derived from resorufin formation was recorded every 5 min for 30 min using FLUOstar OPTIMA® (BMG Labtech, Offenburg, Germany) with excitation and emission filters at 544 and 590 nm, respectively. Co-incubations in which 7-ethoxyresorufin was added together with NADPH at the end of 5 min of pre-warming were also carried out. The IC_{50} value was calculated by nonlinear regression analysis with GraphPad Prism 5.02 (GraphPad Software

Inc., San Diego, CA) using the dose-response with variable slope function.

The kinetics of CYP1A1 inactivation by CBD and its structurally related compounds were determined as described previously [5] with minor modifications. Briefly, all reactions were performed at 37°C in a shaking water bath. Preincubation tubes contained recombinant human CYP1A1 (50 fmol), each CBD-related compound (up to 100 μM), 1.67 mM NADPH, and 50 mM Tris-HCl buffer (pH 7.4) containing 1% fatty acid-free bovine serum albumin in a final volume of 100 μl. The preincubation was performed at 37°C for various amounts of time up to 30 min. An aliquot (20 μl) of the preincubation mixture was transferred to a 180 μl enzyme assay mixture containing 150 nM 7-ethoxyresorufin and 1.67 mM NADPH, and the reaction was initiated. The reaction was terminated after a 20-min incubation by the addition of 200 μl of ice-cold methanol. NADPH was added following the termination of the reaction to obtain a background sample. After being kept on ice for 15 min, 300 μl of the reaction mixture was transferred to a 96-well microtiter plate. Fluorescence was measured using FLUOstar OPTIMA® (BMG Labtech) with the same filters as described above. The observed rates of CYP1A1 inactivation (k_{obs}) were calculated from the initial slopes of the linear regression lines of semilogarithmic plots (remaining EROD activity versus preincubation time). The obtained k_{obs} were plotted against the cannabinoid concentration. The maximal inactivation constant (k_{inact}) and half-maximal inhibitory concentration (K_I) were calculated by nonlinear regression analysis using GraphPad Prism 5.02 (GraphPad Software Inc.).

2.3. Direct inhibition studies

The effects of CBDHQ on CYP1A1 activity were determined using a 96-well microtiter plate as reported previously [5]. The apparent K_i value and mode of inhibition were determined by nonlinear regression analysis with GraphPad Prism 5.02 (GraphPad Software Inc.). Akaike's information criterion was used as a measure of goodness of fit. The mode of inhibition was verified by the visual inspection of Lineweaver-Burk plots of the enzyme kinetic data.

3. Results

3.1 Metabolism-dependent inhibition of human CYP1A1 by compounds structurally related to CBD

The effect of the preincubation on the extent of inhibition achieved by CBD-related compounds was investigated to determine whether these compounds inhibited CYP1A1-mediated EROD activity in a metabolism-dependent manner. A 20-min preincubation of CBD in the presence of NADPH strongly enhanced the inhibition of CYP1A1 activity (Fig. 2), as reported previously [5]. The preincubation of olivetol, which corresponds to the pentylresorcinol moiety of CBD, potentiated the inhibition of CYP1A1 activity. In contrast, no increase was observed in inhibition following the preincubation of *d*-limonene, which corresponds to the terpene moiety of CBD (Fig. 2). Pentylbenzene, which lacks the two free phenolic hydroxyl groups in olivetol, did not potentiate CYP1A1 inhibition (Fig. 2). On the other hand, the inhibition of CYP1A1 activity by the monomethylated and dimethylated derivatives of CBD, CBDM and CBDD, respectively, was augmented by their

preincubation (Fig. 2). The preincubation of CBDV, in which the pentyl side chain of CBD was substituted with a propyl side chain, strongly enhanced CYP1A1 inhibition (Fig. 2). Orcinol, which possesses a methyl group instead of the pentyl group in olivetol, potentiated the inhibition of CYP1A1 activity (Fig. 2). In contrast, resorcinol, which lacks the alkyl side chain of olivetol, did not augment CYP1A1 inhibition (Fig. 2). These results indicated that olivetol, CBDM, CBDD, CBDV, and orcinol as well as CBD inhibited CYP1A1 activity in a metabolism-dependent manner, as shown in Table 1.

3.2. Inactivation of human CYP1A1 activity by compounds structurally related to CBD

To characterize the parameters necessary to inactivate human CYP1A1 by CBD-related compounds, enzyme kinetics were analyzed under the six incubation conditions in which IC_{50} values were decreased due to the preincubation, as shown in Figure 2. In addition, a similar experiment was performed with resorcinol, which did not show any metabolism-dependent inhibition. The preincubation of CBD in the presence of NADPH caused a time- and concentration-dependent decrease in CYP1A1-mediated EROD activity (Fig. 3), as reported previously [5]. CYP1A1 inhibition by all the CBD-related compounds tested, except for resorcinol, was also preincubation time- and concentration-dependent (Fig. 3). As shown in Table 2, the k_{inact} value of olivetol was similar to that of CBD; however, olivetol showed a 10-fold higher K_I value than that of CBD. The K_I values of CBDM and CBDD were 3- and 14-fold higher, respectively, than that of CBD (Table 2) and increased as the number of free phenolic hydroxyl groups decreased. Although the k_{inact} values of CBDM

and CBDD were equivalent, these values were approximately one-third that of CBD (Table 2). The K_I value of CBDV was slightly higher than that of CBD, but was similar to that of CBD (Table 2). The K_I and k_{inact} values of orcinol were 193-fold higher and 6-fold lower, respectively, than those of CBD (Table 2).

3.3. Effects of CBDHQ on human CYP1A1 activity

Bornheim and Grillo [18] previously reported that CBDHQ, a hydroxyquinone metabolite produced from CBD by mouse CYP3A11, was a reactive intermediate that could inactivate CYP3A11. Thus, the effects of CBDHQ on human CYP1A1 activity were investigated to determine whether CBDHQ was a reactive metabolite that could inactivate CYP1A1. The results of the direct inhibition study revealed that CBDHQ competitively inhibited CYP1A1-mediated EROD activity (Fig. 4A). The K_i value of CBDHQ was 11.2 μM, which was 72-fold higher than that of CBD (K_i = 0.155 μM) [5]. The effects of preincubation on CYP1A1 inhibition demonstrated that CBDHQ did not markedly (or significantly) potentiate the inhibition of CYP1A1 activity (Fig. 4B); IC_{50} values for the 0- and 20-min preincubation of CBDHQ were 48.0 and 37.7 μM, respectively.

4. Discussion

Although previous studies reported that CBD inactivated the catalytic activities of human CYP1A1, CYP1A2, CYP1B1, and CYP3A [5, 19], a kinetic analysis has not yet been conducted for CYP3A inactivation. The potency of the CBD-mediated inactivation of

CYP1A1 was shown to be at least 15-fold higher than those of CYP1A2 and CYP1B1, as assessed by the k_{inact}/K_I value [5]. On the other hand, CBD was shown to be a metabolism-dependent, but not mechanism-based inhibitor for CYP2A6 [6]. Furthermore, CBD did not inhibit CYP2B6 [6], CYP2C9 [9], CYP2C19 (unpublished data), or CYP2D6 in a metabolism-dependent manner [8]. These findings indicated that CBD was the most potent inactivator of human CYP1A1 among the CYP enzymes examined so far.

In the present study, we investigated the inhibiting effects of nine CBD-related compounds on CYP1A1 activity to clarify the structural requirements for potent mechanism-based inhibition by CBD. The mechanism-based inhibition of CYP1A1 by olivetol, but not *d*-limonene suggests that the pentylresorcinol structure in CBD may play an important role in this inactivation. The lack of both free phenolic hydroxyl groups in olivetol (i.e. pentylbenzene) abolished the metabolism-dependent inhibition of CYP1A1. Furthermore, inactivation studies on CBDM and CBDD revealed that the methylation of either phenolic hydroxyl group in CBD led to a marked decrease in k_{inact} values. A hydroxyl group at the 2 -position in CBD was equivalent to the hydroxyl group at the 6 -position because of the free rotation of the phenyl ring around the chemical bond at the 1 -position (Fig. 1). These results suggest that both free phenolic hydroxyl groups in CBD may be required for potent inactivation. The substitution of the pentyl group of CBD with a propyl group (i.e. CBDV) did not influence the inhibition of CYP1A1 activity, which implies that carbon atoms at the 4″- and 5″-positions of the pentyl group in CBD were not involved in the inactivation of CYP1A1. The ability to inhibit CYP1A1 activity, even if the side chain in the pentylresorcinol

structure was shortened to a methyl group, was preserved to some extent in orcinol. However, deletion of the side chain (i.e. resorcinol) caused a loss in the ability to inhibit CYP1A1 activity. These results suggest that at least the carbon atom at the 1″-position of the pentyl group in CBD may be required for CYP1A1 inactivation. The results described above suggest that the methylresorcinol structure in CBD may play a key role in CYP1A1 inactivation. One possibility is that the formation of an electrophilic quinone from the methylresorcinol moiety of CBD by human CYP1A1 may lead to CBD-mediated inactivation. However, there is currently no direct evidence that this is involved in the inactivation of CYP1A1.

A previous study reported that CBD inactivated CYP3A-mediated oxidation in human and mouse liver microsomes [19]. CBDHQ may be a reactive metabolite that is produced from CBD by mouse CYP3A11, which ultimately inactivates this enzyme [18]. CBDHQ that formed during the incubation of CBD with mouse CYP3A11 was shown to be trapped as a glutathione adduct [18]. However, we previously demonstrated that glutathione as well as *N*-acetylcysteine did not suppress the inactivation of human CYP1A1 by CBD [5]. Furthermore, superoxide dismutase, a scavenger of reactive oxygen species, did not protect human CYP1A1 from CBD-mediated inactivation. In the present study, the ability of CBDHQ to inhibit human CYP1A1 activity was markedly lower than that of CBD. Therefore, CBDHQ is unlikely to be the reactive intermediate causing CYP1A1 inactivation. However, the reactive intermediate leading to the mechanism-based inhibition of human CYP1A1 by CBD has not yet been identified even though the structure of CBD required for this inactivation is known.

Although CYP1A1 is expressed in various tissues including the liver and lung [20-22], its constitutive expression level is very low. Hepatic and pulmonary CYP1A1 is generally induced following exposure to tobacco smoke [23, 24]. CYP1A1 is known to play a critical role in the metabolic activation of procarcinogens, such as polycyclic aromatic hydrocarbons and heterocyclic amines [22, 25]. The bioactivation of these procarcinogens may induce genotoxicity and carcinogenicity [26]. Therefore, (phyto)chemicals that potently and selectively inhibit CYP1A1 activity are expected to be lead compounds in anticancer chemotherapy. Once a mechanism-based inhibitor inactivates a target enzyme, its inhibitory effect persists until the enzyme is newly synthesized. Thus, it is possible that a mechanism-based inhibitor for CYP1A1 has a higher anti-tumorigenic effect than that of a direct inhibitor for CYP1A1. Although many direct CYP1A1 inhibitors have been identified, there are only a limited number of metabolism-dependent inhibitors and inactivators for human CYP1A1 (Table 3). Rhapontigenin, a stilbene derivative isolated from the rhizome of *Rheum undulatum*, may be a promising anticancer drug because this phytochemical has been shown to potently inactivate human CYP1A1 activity [27]. The potency of the CBD-mediated inactivation of CYP1A1 was similar to that of rhapontigenin, as assessed by k_{inact}/K_I values (Table 3). Based on these findings, CBD may also be a potential lead compound in chemotherapy for the treatment of cancer, especially lung cancer.

5. Conclusions

We demonstrated that several compounds structurally related to CBD as well as CBD

inhibited human CYP1A1 activity. Our results suggest that the methylresorcinol structure in CBD may be required for CYP1A1 inactivation. This study has provided useful information that will lead to a clearer understanding of the precise mechanism(s) underlying the potent CBD inactivation of human CYP1A1.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Footnotes

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Table 1

Summary of the IC_{50} values of CBD and its structurally related compounds for CYP1A1 inhibition with or without preincubation.

Compounds	IC_{50} (μM)		
	Preincubation time		
	0 min (A)	20 min (B)	B/A
CBD	0.671	0.0678	0.101
Olivetol	11.1	2.60	0.234
d-Limonene	> 100	> 100	-
Pentylbenzene	> 100	> 100	-
CBDM	4.12	1.90	0.461
CBDD	29.2	7.68	0.263
CBDV	1.64	0.0677	0.0413
Orcinol	62.3	38.7	0.621
Resorcinol	> 100	> 100	-

All determinations were performed in duplicate.

Table 2

Kinetic parameters for the mechanism-based inhibition of human CYP1A1 by CBD and its structurally related compounds.

Compounds	k_{inact} (min^{-1})	K_I (μM)	k_{inact}/K_I (l $mmol^{-1}$ min^{-1})
CBD	0.215	0.439	490
Olivetol	0.154	4.66	33.0
CBDM	0.0638	1.33	48.0
CBDD	0.0643	6.11	10.5
CBDV	0.226	0.623	363
Orcinol	0.0353	84.7	0.417

Table 3

Summary of the kinetic parameters for the mechanism-based inhibitors of human CYP1A1.

Inactivators	k_{inact} (min^{-1})	K_I (μM)	k_{inact}/K_I ($l\ mmol^{-1}\ min^{-1}$)	Refs.
1-Ethynylpyrene	0.24	0.16	1500	[28]
1-(1-Propynyl)pyrene	0.24	0.16	1500	[28]
Rhapontigenin	0.06	0.09	700	[27]
Chalepensin	0.400	0.64	625	[29]
3 -Flavonepropargylether	0.090	0.24	380	[30]
Emodin compound 1[a]	1.57	5.38	292	[31]
4-(1-Propynyl)biphenyl	0.11	1.5	73	[28]
7-Hydroxyflavone	0.115	2.43	47	[30]
Methylenedioxyphenyl compound[b]	0.034	0.81	42	[32]
Desethylamiodarone	0.03	1.0	30	[33]

[a] 1-Amino-4-chloro-2-methylanthracene-9,10-dione.

[b] 2-[5-Methoxy-2-[5-methyl[2-(3,4-methylenedioxyphenoxy)ethyl]amino]pentyloxy]phenyl]-4-methyl-3-oxo-2H-1,4-benzothiazine.

Figure legends

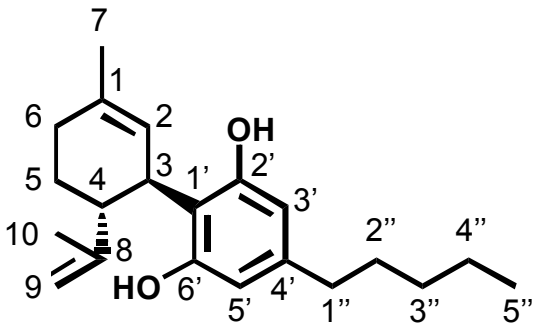
Fig. 1. Chemical structures of CBD and its structurally related compounds.

Fig. 2. Effect of preincubation on the inhibition of human CYP1A1 by CBD and its structurally related compounds. Recombinant human CYP1A1 was preincubated with CBD-related compounds (0 to 100 μM) in the presence of NADPH for 0 or 20 min. EROD activity was measured after the addition of 7-ethoxyresorufin. Each point is the mean of duplicate determinations.

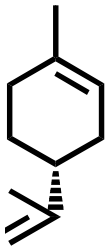
Fig. 3. Inactivation of human CYP1A1 by CBD and its structurally related compounds. Recombinant human CYP1A1 was preincubated with CBD-related compounds (0 to 100 μM) in the presence of NADPH for up to 30 min. Aliquots were removed from the preincubation mixtures at the indicated time points and diluted 10-fold to measure residual activity. In the left graph, each point is the mean of duplicate determinations. The right graph shows the plot of k_{obs} against CBD-related compounds.

Fig. 4. Effects of CBDHQ on human CYP1A1 activity. (A) Recombinant human CYP1A1 was incubated with 7-ethoxyresorufin in the presence of various amounts of CBDHQ. S and v indicate the concentration of 7-ethoxyresorufin (nM) and EROD activity (nmol min^{-1} $nmol^{-1}$ P450), respectively. (B) Recombinant human CYP1A1 was preincubated with CBDHQ (0 to 50 μM) in the presence of NADPH for 0 or 20 min. EROD activity was measured after the

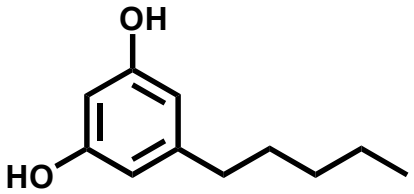
addition of 7-ethoxyresorufin. Each point is the mean of duplicate determinations.



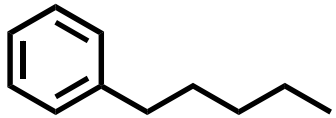
CBD



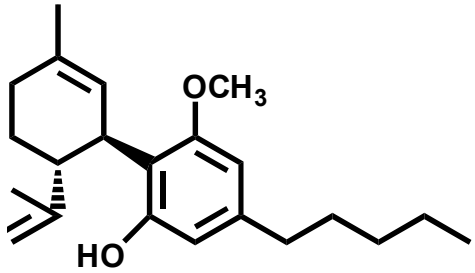
d-Limonene



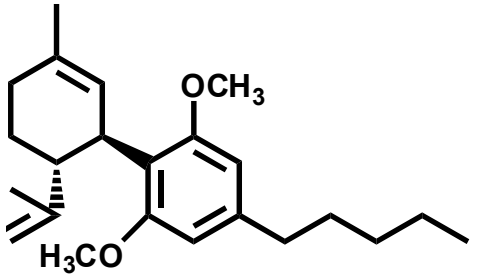
Olivetol



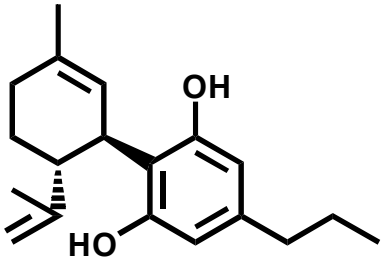
Pentylbenzene



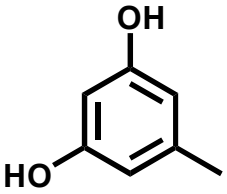
CBDM



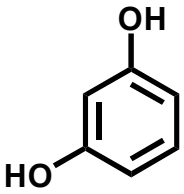
CBDD



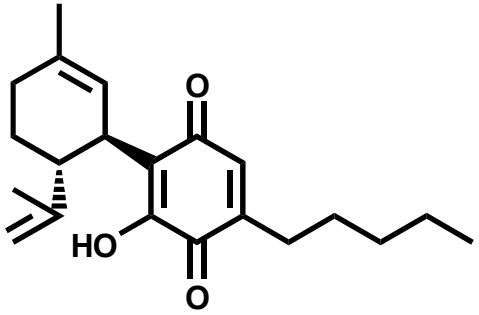
CBDV



Orcinol



Resorcinol



CBDHQ

Fig. 1

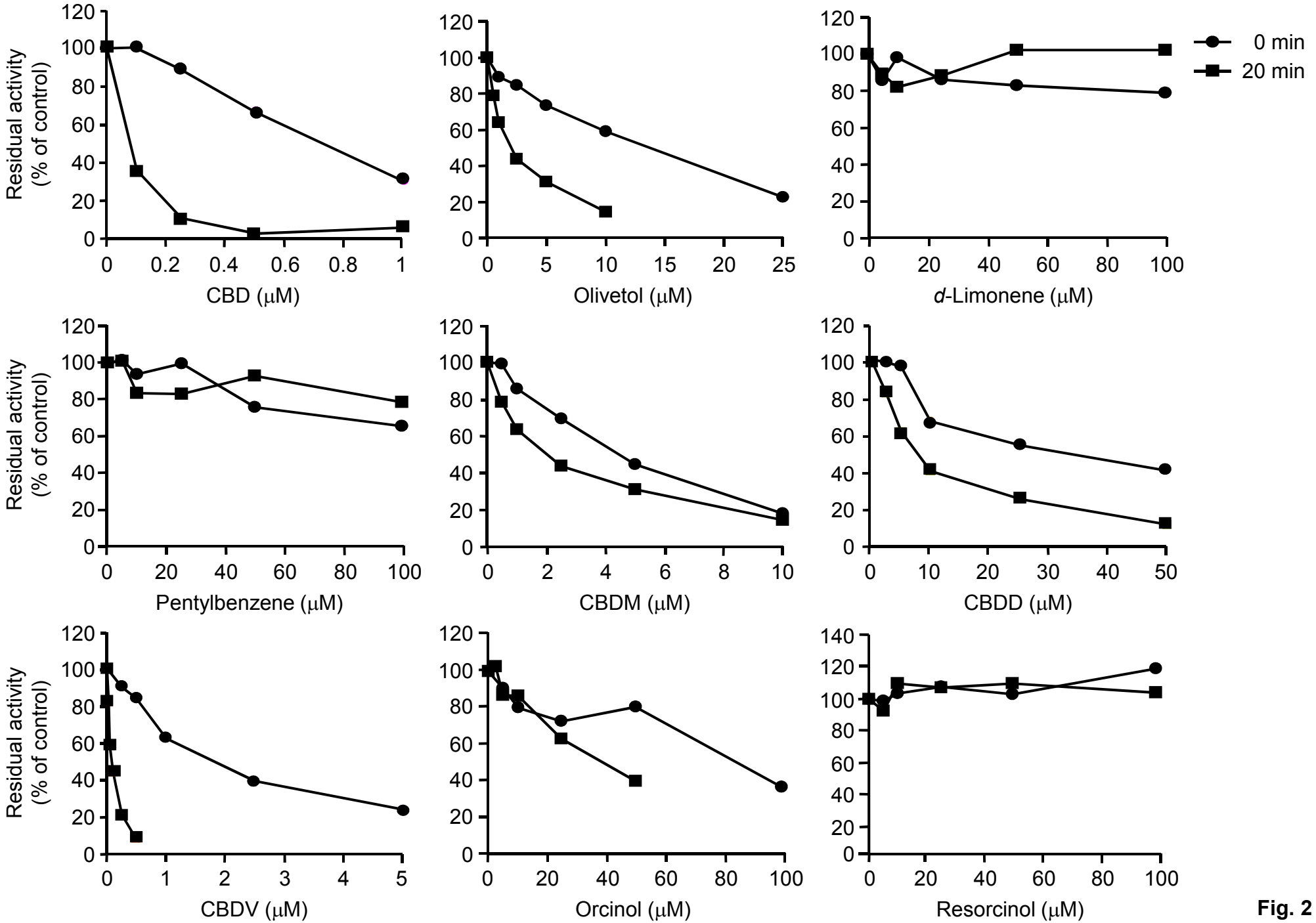

Residual activity (% of control)
CBD (μM)
Olivetol (μM)
d-Limonene (μM)
Pentylbenzene (μM)
CBDM (μM)
CBDD (μM)
CBDV (μM)
Orcinol (μM)
Resorcinol (μM)
0 min
20 min

Fig. 2

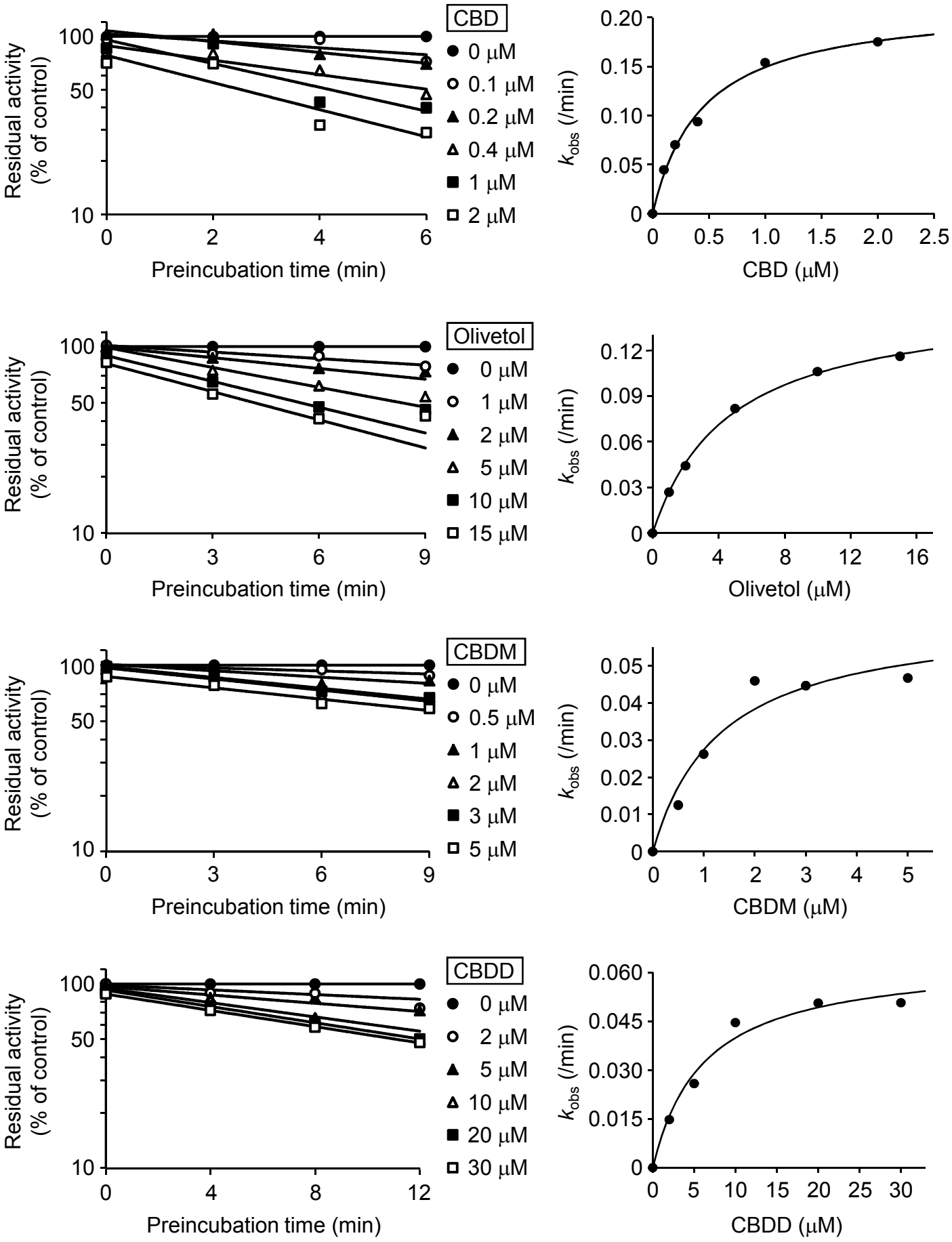

CBD
0 μM
0.1 μM
0.2 μM
0.4 μM
1 μM
2 μM
Residual activity (% of control)
Preincubation time (min)
k_{obs} (/min)
CBD (μM)
Olivetol
0 μM
1 μM
2 μM
5 μM
10 μM
15 μM
Olivetol (μM)
CBDM
0 μM
0.5 μM
1 μM
2 μM
3 μM
5 μM
CBDM (μM)
CBDD
0 μM
2 μM
5 μM
10 μM
20 μM
30 μM
CBDD (μM)

Fig. 3

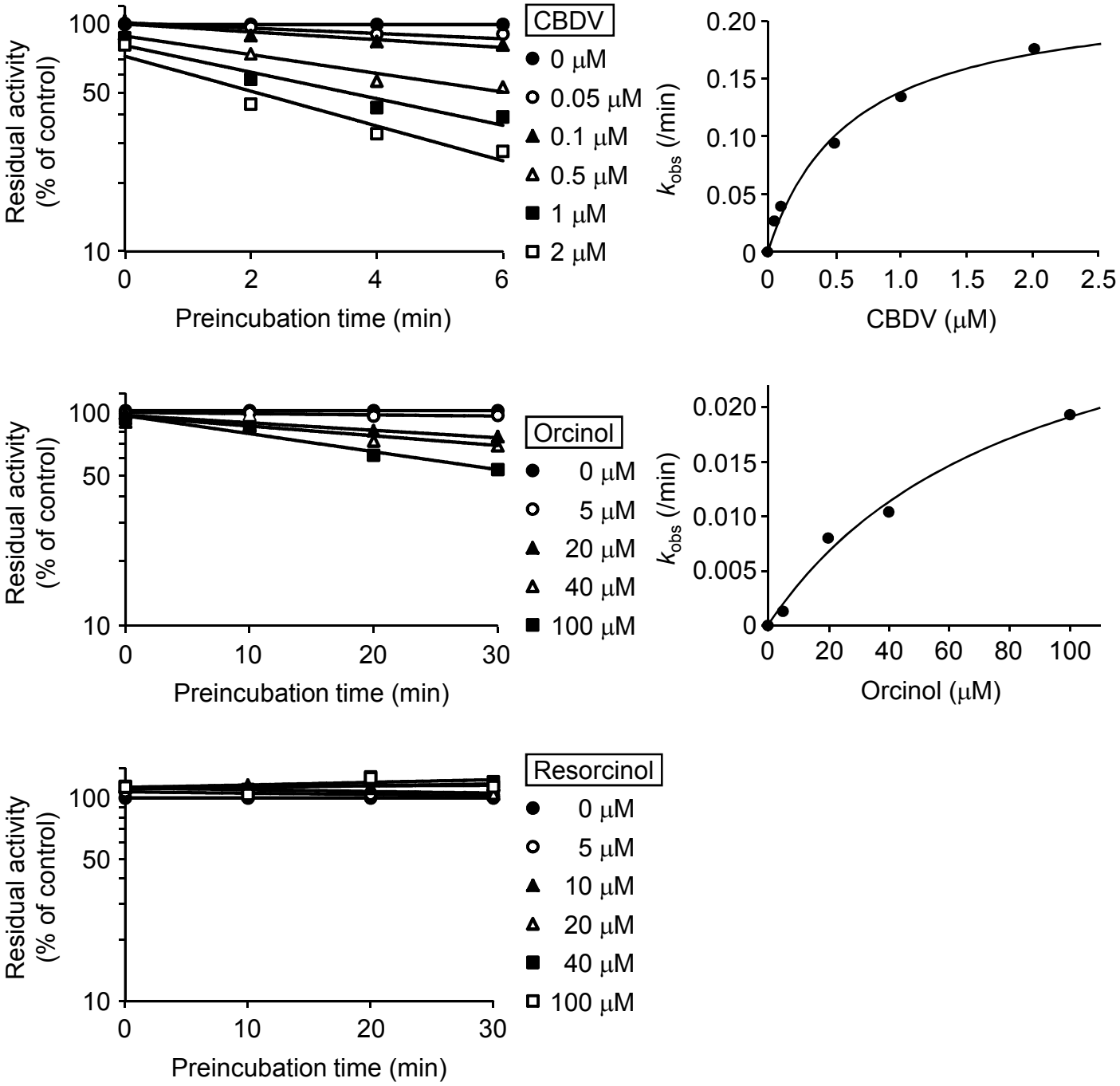


Fig. 3 (continued)

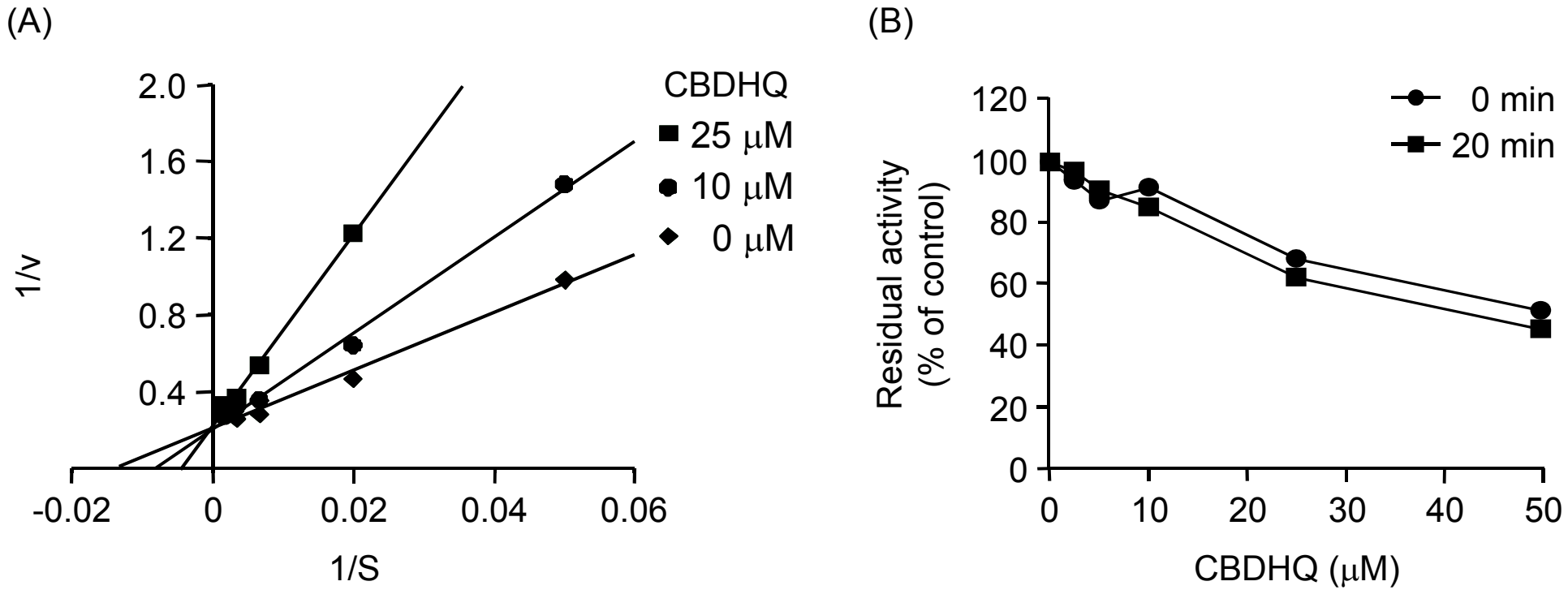

(A)
2.0
1.6
1.2
0.8
0.4
1/v
-0.02
0
0.02
0.04
0.06
1/S
CBDHQ
25 μM
10 μM
0 μM
(B)
120
100
80
60
40
20
0
Residual activity
(% of control)
0
10
20
30
40
50
CBDHQ (μM)
0 min
20 min

Fig. 4