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Cannabichromene, Related Phytocannabinoids, and 5-Fluoro-cannabichromene Have Anticonvulsant Properties in a Mouse Model of Dravet Syndrome

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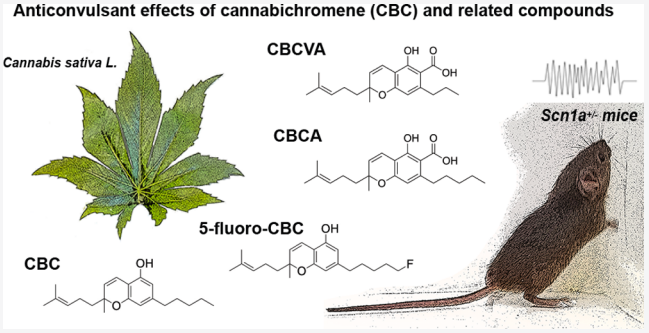
ABSTRACT: Cannabis-based products are increasingly being used to treat refractory childhood epilepsies such as Dravet syndrome. Cannabis contains at least 140 terpenophenolic compounds known as phytocannabinoids. These include the known anticonvulsant compound cannabidiol (CBD) and several molecules showing emergent anticonvulsant properties in animal models. Cannabichromene (CBC) is a phytocannabinoid frequently detected in artisanal cannabis oils used in the community by childhood epilepsy patients. Here we examined the brain and plasma pharmacokinetic profiles of CBC, cannabichromenic acid (CBCA), cannabichromevarin (CBCV), and cannabichromevarinic acid (CBCVA) following intraperitoneal administration in mice. The anticonvulsant potential of each was then tested against hyperthermia-induced seizures in the *Scn1a*$^{+/-}$ mouse model of Dravet syndrome. All phytocannabinoids within the CBC series were readily absorbed and showed substantial brain penetration (brain−plasma ratios ranging from 0.2 to 5.8). Anticonvulsant efficacy was evident with CBC, CBCA, and CBCVA, each significantly increasing the temperature threshold at which *Scn1a*$^{+/-}$ mice had a generalized tonic−clonic seizure. We synthesized a fluorinated derivative of CBC (5-fluoro-CBC), which showed improved brain penetration relative to the parent CBC molecule but not any greater anticonvulsant effect. Since CBC and derivatives are anticonvulsant in a model of intractable pediatric epilepsy, they may constitute part of the mechanism through which artisanal cannabis oils are anticonvulsant in patients.



KEYWORDS: *Cannabichromene, CBC, cannabichromenic acid, CBCA, cannabichromevarin, CBCV, cannabichromevarinic acid, CBCVA, Dravet syndrome*

INTRODUCTION

Epilepsy is a common neurological disease that affects approximately 1% of the population worldwide.[1−3] While current anticonvulsant drugs are effective in most epilepsy patients, 30% of patients are refractory to available treatments.[4] The high prevalence of treatment-resistant epilepsy underscores the need for novel treatment options. The recent interest in cannabis-based medicines for epilepsy has arisen from numerous media stories reporting remarkable improvements in treatment-resistant childhood epilepsy patients using medicinal cannabis. Additionally, the US Food and Drug Administration (FDA), the European Medicines Agency (EMA), and more recently the Therapeutic Goods Administration (TGA) in Australia, approved the purified preparation of cannabidiol (CBD, **1**; Figure 1), Epidiolex, for the treatment of Dravet syndrome and Lennox−Gastaut syndrome.

Although Epidiolex now has regulatory approval for use in treatment-resistant epilepsy, many patients continue to use unregistered cannabis-based products, often reflecting issues of availability and affordability.[5−8] The alternative artisanal or commercially manufactured products often contain an array of phytocannabinoids, and it remains conceivable that compounds in these formulations other than CBD might contribute to their reported anticonvulsant effects.[7] The CBD doses administered in artisanal cannabis-based products are typically much lower than those shown to be effective in reducing seizures in clinical trials with Epidiolex, suggesting involvement of other phytocannabinoids. Moreover, some of the phytocannabinoids found in these products exhibit anticonvulsant properties in animal models. These include cannabidiolic acid (CBDA, **2**), cannabidivarin (CBDV, **3**), Δ^9-

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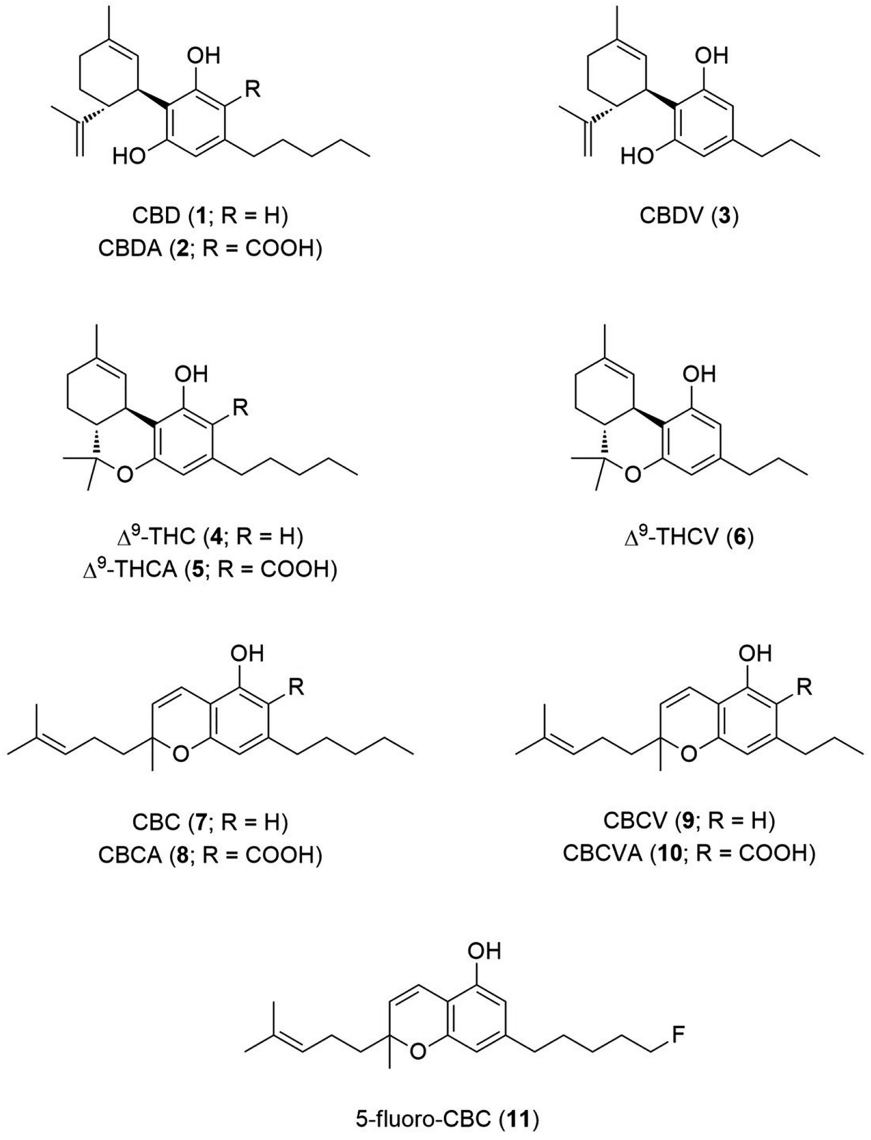

Figure 1. Chemical structures of natural phytocannabinoids and a synthetic derivative.

tetrahydrocannabinol (Δ^9-THC, **4**), Δ^9-tetrahydrocannabinolic acid (Δ^9-THCA, **5**), and Δ^9-tetrahydrocannabivarin (Δ^9-THCV, **6**).[9−16] As such, our laboratory has aimed to systematically mine the cannabis plant for additional, potentially more potent, anticonvulsant phytocannabinoids, with cannabichromene (CBC, **7**) identified as a likely candidate.

In a recent Australian study, CBC was one of the most abundant phytocannabinoids present in the artisanal oils used to treat childhood epilepsy in the community, with only the major phytocannabinoids CBD, CBDA, Δ^9-THC, and Δ^9-THCA found to be more abundant.[7] Notably, in one patient in that study with reported anticonvulsant effects, CBC was administered at a dose as high as 4.7 mg/kg. Further, a recent open-label trial successfully utilized a cannabis herbal extract formulation containing CBC (4%) to treat children with intractable epilepsy.[5] Although the plasma concentrations of CBC were lower than those obtained with CBD in these patients, they were much higher than plasma Δ^9-THC concentrations. Other support for anticonvulsant effects of CBC comes from animal models. In a study using the maximal electroshock (MES) seizure model, CBC displayed a modest anticonvulsant effect by reducing the duration of hindlimb extension at a 25 mg/kg dose.[17] However, in another study using the MES model, CBC at doses up to 500 mg/kg failed to protect mice from seizures.[18]

Conventional induced acute seizure models, such as MES, often fail to identify therapies for treatment-resistant epilepsies, particularly the genetic epileptic encephalopathies of childhood such as Dravet syndrome. Genetic epilepsy models that recapitulate spontaneous epilepsy in humans are etiologically relevant models that can be used in drug discovery. Over 80% of Dravet syndrome patients have a *de novo* loss-of-function mutation in *SCN1A*, the gene that encodes voltage-gated sodium channel $Na_v1.1$.[19] Dravet syndrome typically presents as febrile seizures that progress to spontaneous afebrile seizures.[20] Accordingly, heterozygous deletion of *Scn1a* (*Scn1a*$^{+/-}$) in mice recapitulates the hallmark features of Dravet syndrome, including susceptibility to thermally induced seizures.[21]

The present study assessed the anticonvulsant potential of CBC against hyperthermia-induced seizures in the *Scn1a*$^{+/-}$

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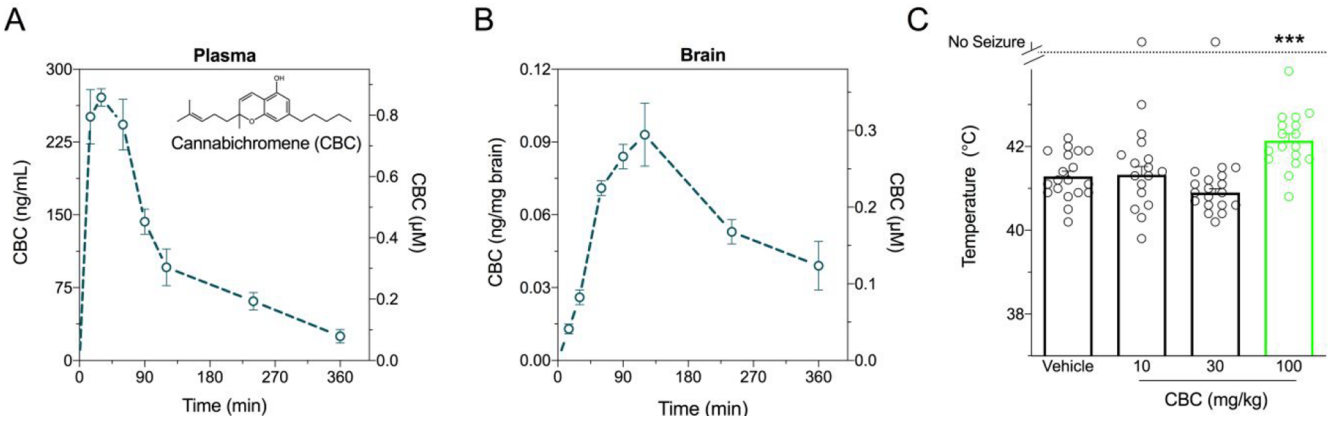

Figure 2. CBC is anticonvulsant against hyperthermia-induced seizures. Concentration–time curves for CBC in (A) plasma and (B) brain following a 10 mg/kg ip injection. Concentrations are depicted as both mass concentrations (left *y*-axis) and molar concentrations (right *y*-axis). Data are expressed as means ± SEM, with n = 4–5 per time point. (A, inset) Chemical structure of cannabichromene. (C) Dose–effect screening of CBC against hyperthermia-induced seizures in *Scn1a*$^{+/-}$ mice. Threshold temperatures of individual mice for generalized tonic–clonic seizure (GTCS) induced by hyperthermia following acute treatment with vehicle or varying doses of CBC. CBC (100 mg/kg) significantly increased the temperature threshold for GTCS, an anticonvulsant effect (green, open symbols). The average temperature of GTCS is depicted by the bar. Error bars represent SEM, with n = 17–19 per group (***p < 0.0005, log-rank Mantel–Cox).

Table 1. Pharmacokinetic Parameters of Cannabichromene Analogues in Mouse Plasma and Brain

	CBC[a]		5-fluoro-CBC[a]		CBCA[a]	
	plasma	brain	plasma	brain	plasma	brain
C (ng/mL)	271 ± 9	93 ± 13[b]	535 ± 76	843 ± 156[b]	8.4 ± 1.4 μg/mL	1.4 ± 0.1 μg/mL[b]
t_{max} (min)	30	120	15	45	30	60
$t_{1/2}$ (min)	98	193	53	83	35	136
AUC (μg·min/mL)	38	32[b]	41	111[b]	606	322[b]
brain–plasma ratio	0.83		2.7		0.53	

	CBCV[a]		CBCVA[a]	
	plasma	brain	plasma	brain
C (ng/mL)	479 ± 52	687 ± 81[b]	20.2 ± 1.7 μg/mL	2.5 ± 0.5 μg/mL[b]
t_{max} (min)	15	30	15	30
$t_{1/2}$ (min)	40	139	34	58
AUC (μg·min/mL)	26	149[b]	840	172
brain–plasma ratio	5.8		0.20	

[a]Dose 10 mg/kg. [b]Brain concentrations (ng/mL) converted from measured concentrations (ng/mg brain) assuming density of 1 g/mL.

mouse model of Dravet syndrome. An important first step was to detail the pharmacokinetic profile of CBC to guide subsequent assessment of its anticonvulsant potential. In addition to CBC, we examined the basic pharmacokinetic profiles and anticonvulsant activity of other CBC-related phytocannabinoids: cannabichromenic acid (CBCA, **8**), cannabichromevarin (CBCV, **9**), cannabichromevarinic acid (CBCVA, **10**), and a terminally fluorinated synthetic analogue of CBC (5-fluoro-CBC, **11**). CBCA and CBCVA are formed enzymatically in the cannabis plant; therefore, they are relatively abundant in raw cannabis material and cold-extracted cannabis products.[22] The neutral phytocannabinoids, CBC and CBCV, are formed via decarboxylation of the respective acid precursor as a result of light and heat exposure.

RESULTS AND DISCUSSION

Pharmacokinetic Profile of CBC in Mouse Plasma and Brain. Intraperitoneal (ip) administration of CBC resulted in rapid absorption (plasma t_{max} 30 min) and a relatively long half-life ($t_{1/2}$ = 98 min; Figure 2, Table 1). Distribution into brain tissue was delayed relative to plasma, not reaching t_{max} until 120 min (Figure 2B). CBC exhibited a longer half-life ($t_{1/2}$ = 193 min) in brain than in plasma. Total exposure of CBC in brain tissue was nearly equivalent to that of plasma as determined by AUC values (brain–plasma ratio 0.83).

CBC Is Anticonvulsant against Hyperthermia-Induced Seizures in *Scn1a*$^{+/-}$ Mice. CBC was evaluated for efficacy against thermally induced seizures in *Scn1a*$^{+/-}$ mice. Between postnatal day 14 and 16, *Scn1a*$^{+/-}$ mice were treated with a single ip injection of vehicle or CBC and challenged with a thermal event. CBC was anticonvulsant against hyperthermia-induced seizures (Figure 2C, Table 2). A significant increase in GTCS temperature threshold was observed with 100 mg/kg CBC (p = 0.0002). By comparison, the effective anticonvulsant dose of CBD against hyperthermia-induced seizures is also 100 mg/kg.[10,23,24]

Fluorination of CBC Achieves Greater Brain Penetration than CBC. We hypothesized that the anticonvulsant efficacy of CBC might be improved by enhanced brain penetration. To this end, a fluorinated derivative of CBC was synthesized, 5-fluoro-CBC, that was predicted to have reduced lipophilicity and better brain penetration. The synthesis of CBC and 5-fluoro-CBC is shown in Figure 3. Citral (**12**) was condensed with either olivetol (**13**) or 5-(5-fluoropentyl)-

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Table 2. Plasma Cannabinoid Concentrations in Experimental *Scn1a*$^{+/-}$ Mice

cannabinoid	dose (mg/kg)	plasma concentration (μg/mL)
CBC	10	394 ± 128 ng/mL
	30	4.1 ± 0.2
	100	12.4 ± 0.8
CBCA	10	15.8 ± 1.2
	30	53.7 ± 6.3
	100	95.7 ± 18.4
CBCV	10	1013 ± 52 ng/mL
	30	2.6 ± 0.4
	100	8.9 ± 1.3
CBCVA	10	24.1 ± 4.8
	30	102.3 ± 11.2
	100	157.7 ± 16.1
5-fluoro-CBC	10	714 ± 79 ng/mL
	30	1.9 ± 0.4
	100	8.2 ± 0.9

resorcinol (**14**) in the presence of catalytic ethylenediamine diacetate to produce CBC or 5-fluoro-CBC, respectively.

5-fluoro-CBC was rapidly absorbed in plasma (t_{max} = 15 min; Figure 4A, Table 1) and brain (t_{max} = 45 min; Figure 4B). The simple substitution of fluorine caused striking improvements in plasma and brain absorption compared to those of CBC, which had t_{max} values in plasma and brain of 30 and 120 min, respectively. The C_{max} (843 ± 156 ng/mL) achieved in the brain was significantly greater than the plasma C_{max} (543 ± 76 ng/mL). Total exposure of 5-fluoro-CBC in brain tissue was nearly three times that of the plasma (brain−plasma ratio 2.7), confirming the addition of the fluorine increased the brain penetration as predicted. These results are consistent with reports that an appropriately placed single fluorine substitution can dramatically improve absorption, distribution, metabolism, and excretion (ADME) properties of drugs.[25,26]

The effect of 5-fluoro-CBC on hyperthermia-induced seizures in *Scn1a*$^{+/-}$ mice was then evaluated. The 100 mg/kg dose of 5-fluoro-CBC was found to be anticonvulsant, significantly elevating the GTCS temperature threshold compared to vehicle (p = 0.0376, Figure 4C). Despite 5-fluoro-CBC exhibiting substantially increased brain penetration compared to CBC, it did not show greater anticonvulsant efficacy. Both CBC and 5-fluoro-CBC were anticonvulsant against hyperthermia-induced seizures at 100 mg/kg. Since the brain−plasma ratio for 5-fluoro-CBC was nearly three times that of CBC, it was hypothesized that it would be more potent with perhaps a 30 mg/kg dose having anticonvulsant effects. It is possible that the introduction of the fluorine disrupted engagement with the molecular target(s) responsible for the anticonvulsant effect of CBC. However, in the absence of knowing the anticonvulsant mode of action(s), it is difficult to reach any firm conclusions.

Pharmacokinetic Profiles of Other CBC-Related Phytocannabinoids: CBCA, CBCV, and CBCVA. The pharmacokinetic profiles of three CBC-related phytocannabinoids (CBCA, CBCV, and CBCVA) were then characterized in mouse plasma and brain (Figure 5A).

For CBCA, a high maximal plasma concentration (C_{max} = 8.4 ± 1.4 μg/mL; Figure 5B, Table 1) was observed 30 min after ip injection of 10 mg/kg. Distribution into brain tissue was delayed, reaching t_{max} at 60 min (Figure 5C). CBCA exhibited a long half-life ($t_{1/2}$ = 136 min) in brain compared to 35 min in plasma. Although overall drug exposure of CBCA in brain tissue was lower than that in plasma, CBCA had appreciable brain penetration (brain−plasma ratio 0.53). This substantial brain penetration of CBCA observed here is in contrast to our previous study where CBCA was not detectable in brain tissue following a 5 mg/kg ip injection.[9] An important difference is that in the previous study vegetable oil was used as a vehicle, while here an ethanol−Tween80−saline (ratio 1:1:18) vehicle was used. A similar result was observed for CBDA where its brain−plasma ratio was significantly increased in a Tween-based vehicle compared to an oil vehicle.[9]

For CBCV, rapid absorption was observed with a t_{max} of 15 min in plasma and 30 min in brain (Figure 5D,E; Table 1). Elimination from plasma was rapid ($t_{1/2}$ = 40 min); however, a long half-life ($t_{1/2}$ = 139 min) was observed in brain, which led to a greater AUC value for brain compared to plasma. Total exposure of CBCV in brain tissue was nearly six times that of plasma (brain−plasma ratio 5.8).

Similarly, CBCVA was rapidly absorbed with a plasma t_{max} of 15 min and a half-life of 34 min (Figure 5F, Table 1). CBCVA achieved high concentrations in the brain (C_{max} = 2.5 ± 0.5 ng/mg brain); however, its plasma C_{max} (20.2 ± 1.7 μg/mL) was significantly higher (Figure 5G). The brain−plasma ratio of CBCVA was 0.20.

CBCA and CBCVA Are Anticonvulsant against Hyperthermia-Induced Seizures in *Scn1a*$^{+/-}$ Mice. Finally, the anticonvulsant efficacy of CBCA, CBCV, and CBCVA was determined against thermally induced seizures in *Scn1a*$^{+/-}$ mice. Compared to vehicle, a significant elevation in GTCS temperature threshold was observed with 100 mg/kg CBCA (p = 0.0001; Figure 6A). CBCV had no effect on hyperthermia-induced seizures at any dose tested despite its substantial brain uptake (Figure 6B). Its acid form, CBCVA, however, was anticonvulsant against thermally induced seizures with 100 mg/kg significantly elevating the temperature threshold for GTCS compared to vehicle (p = 0.0365; Figure 6C).

Brain and Plasma Pharmacokinetic Parameters of CBC-Related Compounds in Mice. The current study presents the first pharmacokinetic data for CBC, 5-fluoro-CBC, CBCV, and CBCVA in mice. Pharmacokinetic parameters have

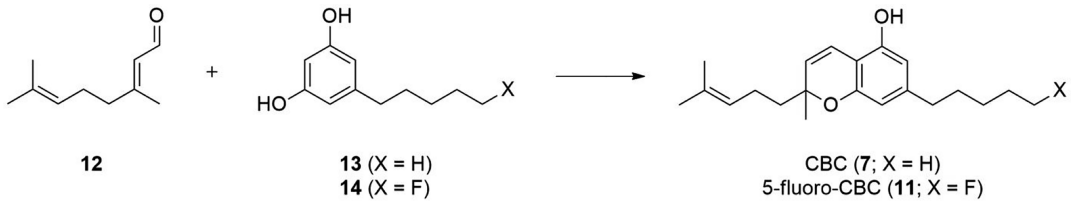

Figure 3. Synthesis of CBC (7) and 5-fluoro-CBC (**11**). Reagents and conditions: ethylenediamine diacetate (25 mol %), toluene, 110 °C, 6 h; yield 35% (7) and 31% (**11**).

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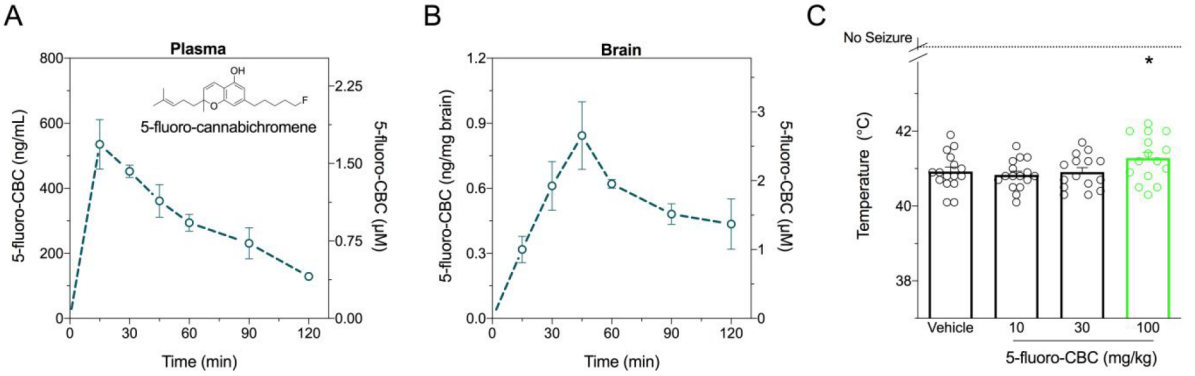

Figure 4. 5-fluoro-CBC exhibits substantial brain penetration. Concentration−time curves for 5-fluoro-CBC in (A) plasma and (B) brain following a 10 mg/kg ip injection. Concentrations are depicted as both mass concentrations (left *y*-axis) and molar concentrations (right *y*-axis). Data are expressed as means ± SEM, with $n = 4$ per time point. (A, inset) Chemical structure of 5-fluoro-CBC. (C) Dose−effect screening of 5-fluoro-CBC against hyperthermia-induced seizures in *Scn1a*$^{+/-}$ mice. Threshold temperatures of individual mice for generalized tonic−clonic seizure (GTCS) induced by hyperthermia following acute treatment with vehicle or varying doses of 5-fluoro-CBC. 5-Fluoro-CBC (100 mg/kg) significantly increased the temperature threshold for GTCS, indicating an anticonvulsant effect (green, open symbols). The average temperature of GTCS is depicted by the bar. Error bars represent SEM, with $n = 15-16$ per group (*$p < 0.05$, log-rank Mantel−Cox).

been characterized previously for CBCA; however, here a Tween-based vehicle was used. In general, the phytocannabinoids within this CBC series were rapidly absorbed in plasma following ip injection with plasma t_{max} values ≤30 min. Absorption into brain tissue tended to be slightly delayed compared to plasma, with t_{max} values between 30 and 120 min. These absorption rates are consistent with those of other phytocannabinoids.[9,27] Interestingly, while the half-lives of each in plasma were relatively short (34−98 min), brain half-lives were substantially longer. The half-lives in brain ranged from 1.5 to 3.5 times longer than the plasma half-lives. This is likely due to the lipophilic nature of these compounds, which tend to store in lipid-rich tissues like the brain.

Overall, there was good brain penetration within the CBC series, with CBC exhibiting a brain−plasma ratio of 0.83. By addition of a fluorine to CBC (5-fluoro-CBC), the brain−plasma ratio nearly tripled (2.7). The increased permeability is not surprising as fluorine substitutions are a common strategy employed in drug discovery and development to increase blood−brain barrier (BBB) penetration.[28] Surprisingly, CBCA and CBCVA had fair brain penetration (brain−plasma ratios of 0.53 and 0.20, respectively) despite containing a carboxylic acid functional group. Drugs with a carboxylic acid group are negatively charged at physiological pH, which hampers passive diffusion across the BBB. Nonsedative antihistamines purposefully contain a carboxylic acid moiety to restrict them peripherally.[29]

In a previous study, CBCA was not detectable in brain tissue when administered in a vegetable oil vehicle.[9] Here, when CBCA was administered in a Tween-based vehicle, its brain penetration increased substantially. Increased brain permeability with a Tween-based vehicle is not unusual. Several reports in the literature describe increased biomembrane permeability and altered pharmacokinetic profiles of drugs when administered with nonionic surfactants, including Tween80.[30−33] In fact, the brain−plasma ratio of CBDA was nearly 50 times greater in a Tween-based vehicle compared to a vegetable oil vehicle (brain−plasma ratios of 1.9 and 0.04, respectively).[9] Nonionic surfactants have been shown to inhibit P-glycoprotein (P-gp)-mediated transport, and since some phytocannabinoids are known P-gp substrates, it is possible that CBCA is also a substrate.[34−37] In this case, Tween80 could inhibit its efflux from the brain; future studies could investigate this possibility.

The Discovery of CBC-Related Phytocannabinoids with Novel Anticonvulsant Activity in a Mouse Model of Intractable Childhood Epilepsy. In addition to characterizing the pharmacokinetic parameters of the phytocannabinoids within the CBC series, we examined the effect of each on hyperthermia-induced seizures in the *Scn1a*$^{+/-}$ mouse model of Dravet syndrome. CBC was anticonvulsant and significantly increased the temperature threshold of GTCS. This is in contrast to two previous studies in mice that reported CBC lacked anticonvulsant efficacy in the conventional MES seizure model, as CBC (up to 500 mg/kg) did not protect against generalized seizures.[18] Although, one study reported hints of anticonvulsant activity, with CBC modestly reducing the duration of MES-induced hindlimb extension, albeit not in a dose-dependent manner.[17] Taken together with the present findings, this might imply that CBC has preferential anticonvulsant potential against childhood epileptic encephalopathies.

The fluorination of CBC, generating 5-fluoro-CBC, increased the brain−plasma ratio by nearly 3-fold compared to CBC itself. Despite significantly increasing the brain−plasma permeability, 5-fluoro-CBC was no more potent than CBC against hyperthermia-induced seizures. The observations that CBC and its fluorinated derivative were anticonvulsant at the same dose as CBD against thermally induced seizures suggest some promise in pursuing these compounds clinically.[10,23,24] Additionally, we identified two novel anticonvulsant phytocannabinoids, CBCA and CBCVA, that were also both effective at 100 mg/kg.

Future studies might usefully probe the anticonvulsant mode(s) of action of this CBC series. Knowledge on the pharmacological activity is extremely limited for CBC and virtually nonexistent for the other phytocannabinoids such as CBCA, CBCV, and CBCVA. Recently, CBC has been identified as a CB_2 receptor agonist, which could be relevant to the anticonvulsant effects observed here.[38] In a pentylenetetrazole (PTZ)-induced seizure model, genetic deletion of CB_2 receptors increased seizure susceptibility.[39] Furthermore, CB_2 receptor expression appears to be increased in lymphocytes of Dravet syndrome patients.[40] Importantly, CBC has little

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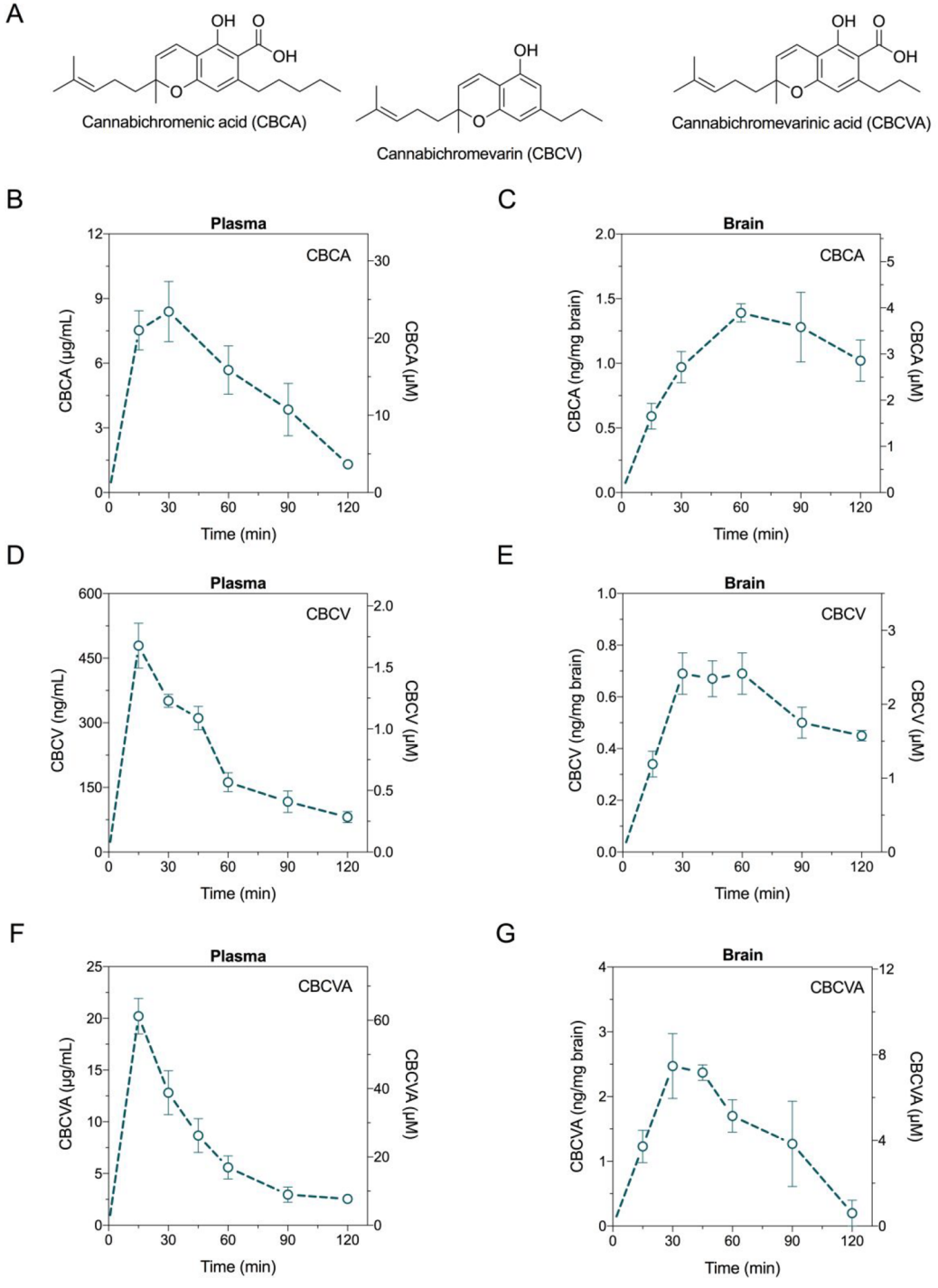

Figure 5. Pharmacokinetic analysis of CBCA, CBCV, and CBCVA in mouse plasma and brain samples. (A) Chemical structures of the phytocannabinoids cannabichromevarin (CBCV), cannabichromenic acid (CBCA), and cannabichromevarinic acid (CBCVA). Concentration–time curves for CBCV in (B) plasma and (C) brain, CBCA in (D) plasma and (E) brain, and CBCVA in (F) plasma and (G) brain following 10 mg/kg ip injections. Concentrations are depicted as both mass concentrations (left *y*-axis) and molar concentrations (right *y*-axis). Data are expressed as means ± SEM with $n = 4$ per time point.

activity at CB_1 receptors so is unlikely to produce THC-like psychotropic effects.[38] CBC has also been reported to be a potent agonist at the transient receptor potential ankyrin 1 (TRPA1) channel (EC_{50} = 60 nM).[41] However, long-term activation of TRPA1 channels exacerbated seizure activity in PTZ-induced kindled rats.[42]

Other molecular targets of the more extensively studied phytocannabinoid CBD might also apply to CBC and could be explored in future studies. Epilepsy-relevant targets might include voltage-gated sodium and calcium channels, transient receptor potential vanilloid type 1 (TRPV1), and $GABA_A$ channels, as well as GPR55 and 5-HT1A receptors.[24,43−48] Moreover, CBC metabolites, which have been characterized in several species including mice, could be examined to observe whether they contribute to the anticonvulsant effects of CBC.[49]

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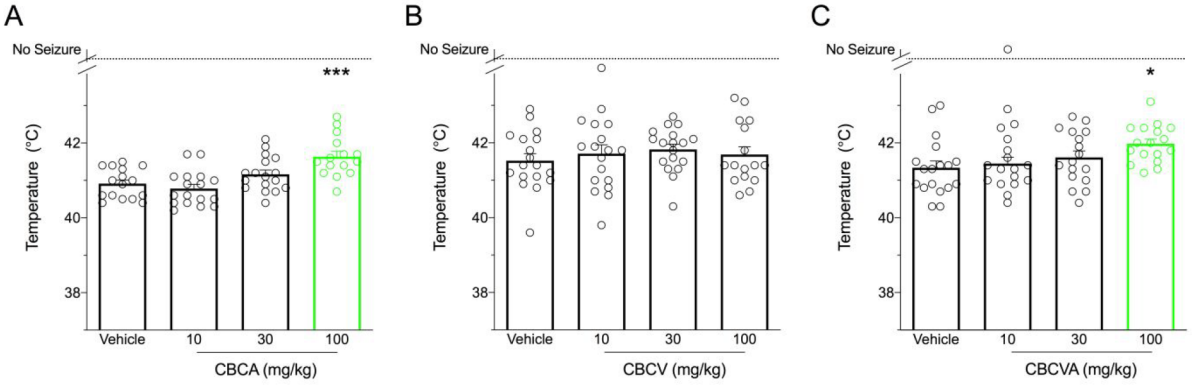

Figure 6. Dose–effect screening of CBCA, CBCV, and CBCVA using hyperthermia-induced seizures in *Scn1a*$^{+/-}$ mice. Threshold temperatures of individual mice for generalized tonic–clonic seizure (GTCS) induced by hyperthermia following acute treatment with varying doses of (A) CBCA, (B) CBCV, or (C) CBCVA. CBCA (100 mg/kg) and CBCVA (100 mg/kg) significantly increased the temperature threshold for GTCS, indicating anticonvulsant effects (green, open symbols). CBCV had no effect on thermally induced seizures. The average temperature of GTCS is depicted by the bar. Error bars represent SEM with n = 15–18 per group (*p < 0.05 and ***p < 0.0005, log-rank Mantel–Cox).

CONCLUSION

Here, the brain and plasma pharmacokinetic profiles of CBC and related phytocannabinoids were determined and could be used to guide future *in vivo* experiments with these compounds. CBC, 5-fluoro-CBC, CBCA, and CBCVA all exhibited anticonvulsant properties against hyperthermia-induced seizures in the *Scn1a*$^{+/-}$ mouse model of Dravet syndrome. Further studies could explore whether these cannabinoids are also anticonvulsant against spontaneous seizures or prolong the lifespan of *Scn1a*$^{+/-}$ mice.

METHODS

Chemicals and Reagents. All reagents, reactants, and solvents were purchased from Sigma-Aldrich (St. Louis, USA) or WuXi AppTec (Shanghai, CHN) and used as purchased. CBCV was purchased from Epichem (Bentley, AUS). CBC and 5-fluoro-CBC were synthesized as described below. CBCA and CBCVA were generously provided by Professor Michael Kassiou at the University of Sydney. The purity of all the cannabinoids used were CBC (>95%), CBCA (>99%), CBCV (>96%), and CBCVA (>95%). When available, analytical standards were purchased from Novachem Pty Ltd. (Heidelberg West, AUS).

Animals. All animal care and experimental procedures were approved by the University of Sydney Animal Ethics Committee, and all procedures were in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. *Scn1a*$^{+/-}$ mice were purchased from The Jackson Laboratory (stock 37107-JAX; Bar Harbor, USA) and maintained as a congenic line on the 129S6/SvEvTac background (129.*Scn1a*$^{+/-}$). For experiments, F1 mice were generated by breeding heterozygous 129.*Scn1a*$^{+/-}$ mice with wild-type C57BL/6J (Jackson Laboratory stock 000664; Animal Resources Centre; Canning Vale, AUS). The *Scn1a* genotype was determined as previously described.[50]

Pharmacokinetic Studies. Drugs were prepared fresh on the day of the experiment as solutions in ethanol/Tween-80/saline (1:1:18). CBCA (100 mg/kg) was a suspension, with CBCV (100 mg/kg) near its solubility limit. Drugs were administered as an intraperitoneal (ip) injection in a volume of 10 mL/kg. Wild-type male and female mice (postnatal day 21–28, P21–28) received a single ip injection of 10 mg/kg CBC, 5-fluoro-CBC, CBCA, CBCV, or CBCVA. At selected time points (15–120 min), mice were anesthetized with isoflurane and whole blood was collected by cardiac puncture. Plasma was isolated by centrifugation (9000*g* for 10 min, 4 °C) and stored at −80 °C until assayed. Whole brain was also collected and stored at −80 °C. Each time point (n = 4–5) contained at least one male and one female per group.

Sample Preparation. Plasma samples (50 μL, 5 μL for CBCA and CBCVA) were spiked with diazepam (2 μg/mL) as an internal standard, and protein precipitation was achieved by vortex-mixing with 200 μL of acetonitrile. The organic layer was isolated by centrifugation (4000*g* for 10 min) and evaporated to dryness with N_2. Samples were reconstituted in acetonitrile (90 μL) and 0.1% formic acid in water (300 μL) for supported-liquid extraction (SLE) with methyl *tert*-butyl ether (MTBE) using Biotage Isolute SLE+ columns (Uppsala, SWE). Samples were evaporated to dryness with N_2 and reconstituted in acetonitrile and 0.1% formic acid in water (1:1, v/v) for analysis. Brains were prepared as described previously.[10] Briefly, each half brain was homogenized in 5× volume of acetonitrile. Homogenates were centrifuged (20000*g* for 30 min, 4 °C), and brain supernatants were spiked with diazepam (10 μg/mL). Extraction was achieved by vortex-mixing with 3× volume of ice-cold acetonitrile for 10 min. The organic layer was isolated by centrifugation (20000*g* for 15 min, 4 °C). Supernatants were filtered through Amicon Ultracel-3K (Merck-Millipore; Burlington, USA) filtration devices. Filtrates were evaporated to dryness with N_2 and underwent SLE extraction with MTBE and reconstitution for analysis as described above.

Analytical Methods. Samples were assayed by LC-MS/MS using a Shimadzu Nexera ultra-HPLD coupled to a Shimadzu 8030 triple quadrupole mass spectrometer (Shimadzu Corp.; Kyoto, JPN) as previously described.[9,10] The mass spectrometer was operated in positive electrospray ionization mode (negative mode for CBCVA) with multiple reaction monitoring. Details regarding MS conditions for each cannabinoid are provided in Table 3. Quantification of cannabinoids in plasma and brain samples was performed by comparing samples to standards prepared with known amounts of drug.

Pharmacokinetic Calculations. Plasma and brain cannabinoid concentrations at each time point were averaged, and pharmacokinetic parameters were calculated by noncompartmental analysis. Elimination rate constants were determined by linear regression of the terminal component of the concentration–time curve using GraphPad Prism 7.2 (La Jolla, USA). The log–linear trapezoidal method was used to calculated total drug exposure (area under concentration–time curve) using equations described previously.[21]

Hyperthermia-Induced Seizures. Hyperthermia-induced seizure experiments were conducted on male and female *Scn1a*$^{+/-}$ mice at P14–16 as previously described with n = 15–19 per group (each sex accounted for approximately one-half of each group).[21,23] Briefly, mice were pretreated with vehicle or cannabinoid by a single ip injection. Fifteen minutes prior to the target experimental (postdose) time point for each drug, the rectal probe was inserted, mice acclimated for 5 min, and the hyperthermia protocol was initiated. Following the hyperthermia-induced seizure protocol, plasma samples were isolated and stored at −80 °C until assayed. Seizure threshold

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Table 3. Parameters for LC-MS/MS Detection of Cannabinoids

compound	molecular weight	parent > daughter ions (m/z)	plasma LOQ[a] (μg/mL)	brain LOQ[a] (ng/mg brain)
CBC	314.5	315.20 > 193.25	0.01−20	0.03−0.25
		315.20 > 81.25		
5-fluoro-CBC	333.2	333.45 > 277.35	0.01−10	0.05−1
		333.45 > 211.10		
CBCA	358.4	359.10 > 341.25	0.005- 10	0.05−4
		359.10 > 316.00		
CBCV	286.4	287.00 > 165.15	0.01−10	0.05−1
		287.00 > 231.20		
		287.00 > 81.15		
CBCVA	330.4	329.35 > 163.25	0.01−20	0.05−4
		329.35 > 311.30		
		329.35 > 285.20		

[a]LOQ, Limit of Quantification. Range from lower to upper limits.

temperatures were compared using Mantel−Cox log-rank test, and $p < 0.05$ was considered statistically significant. No significant sex differences were observed, so groups were combined across sex.

Experimental time points were based on determined time-to-peak plasma and brain concentrations from our pharmacokinetic studies. Experimental time points used were as follows: 45 min, 5-fluoro-CBC; 30 min, CBC and CBCA; 15 min, CBCV and CBCVA, which were administered after the 5 min acclimation period.

Synthesis of Cannabichromene (CBC) and 5-Fluoro-cannabichrome (5-fluoro-CBC). All reactions were performed under an atmosphere of nitrogen unless otherwise specified. Analytical thin-layer chromatography was performed using Merck aluminum-backed silica gel 60 F254 (0.2 mm) plates (Merck; Darmstadt, GER), which were visualized using shortwave (254 nm) UV fluorescence. Flash chromatography was performed using a Biotage Isolera Spektra One and Biotage SNAP KP-Sil silica cartridges, with gradient elution terminating at the solvent combination indicated for each compound. Nuclear magnetic resonance spectra (NMR) were recorded at 298 K using an Agilent 400 MHz spectrometer. The data are reported as chemical shift (δ ppm) relative to the residual protonated solvent resonance, multiplicity (s = singlet, br s = broad singlet, d = doublet, br d = broad doublet, t = triplet, q = quartet, app quin. = apparent quintet, m = multiplet), coupling constants (J Hz), relative integral, and assignment. Assignment of carbon signals for the parent compounds was assisted by correlation spectroscopy (COSY), distortionless enhancement by polarization transfer (DEPT), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple-bond correlation (HMBC) experiments where necessary.

General Procedure for the Synthesis of CBC and 5-fluoro-CBC. Using a modified procedure, a flame-dried reaction vessel was charged with the appropriate resorcinol (5 mmol, 1 equiv), citral (**1**, 1.09 mL, 6 mmol, 1.2 equiv), ethylenediamine diacetate (225 mg, 1.25 mmol, 0.25 equiv), and toluene (50 mL).[51] The solution was heated at reflux for 6 h before being cooled to ambient temperature, and the solvent was removed under reduced pressure. The resulting crude oils were purified via flash column chromatography.

*2-Methyl-2-(4-methylpent-3-en-1-yl)-7-pentyl-2H-chromen-5-ol (CBC, **7**).* Subjecting olivetol (**13**, 900 mg, 5 mmol) to the general procedure gave, following purification by flash column chromatography, the title compound (556 mg, 35%) as a yellow oil. R_f = 0.74 (hexanes/EtOAc, 80:20); NMR (400 MHz, CD_3OD) δ 6.65 (dd, J = 10.1 Hz, 1H), 6.17 (d, J = 1.5 Hz, 1H), 6.10 (d, J = 1.5 Hz, 1H), 5.47 (d, J = 10.1 Hz, 1H), 5.15−5.11 (m, 1H), 92.47−2.41 (m, 2H), 2.17−2.06 (m, 2H), 1.67 (s, 3H), 1.67−1.63 (m, 2H), 1.66−1.53 (m, 2H), 1.58 (s, 6H), 1.39−1.29 (m, 4H), 1.35 (s, 3H), 0.92 (t, J = 7.1 Hz, 3H) ppm. All spectral data is consistent with that previously reported (Figure S1, S2).[52]

*7-(5-Fluoropentyl)-2-methyl-2-(4-methylpent-3-en-1-yl)-2H-chromen-5-ol (5-Fluoro-CBC, **11**).* Subjecting 5-(5-fluoropentyl)-benzene-1,3-diol (**14**, 327 mg, 1.65 mmol) to the general procedure gave, following purification by flash column chromatography, the title compound (158 mg, 31%) as a yellow oil. R_f = 0.76 (hexanes/EtOAc, 80:20); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.61 (d, J = 10.0 Hz, 1H), 6.25 (s, 1H), 6.11 (s, 1H), 5.50 (d, J = 10.0 Hz, 1H), 5.12−5.07 (m, 1H), 4.72 (brs, 1H), 4.43 (dt, J = 47.3, 6.2 Hz, 2H), 2.49−2.45 (m, 2H), 2.16−2.05 (m, 2H), 1.77−1.67 (m, 3H), 1.67 (s, 3H), 1.66−1.58 (m, 3H), 1.57 (s, 3H), 1.46−1.39 (m, 2H), 1.38 (s, 3H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 154.3, 151.2, 144.3, 131.8, 127.5, 124.3, 116.9, 109.3, 107.8, 107.2, 84.2 (d, $^1J_{C-F}$ = 164.1 Hz), 78.4, 41.2, 35.9, 30.7, 30.4 (d, $^2J_{C-F}$ = 19.5 Hz), 26.4, 25.8, 25.0 (d, $^3J_{C-F}$ = 5.5 Hz), 22.9, 17.8 ppm; IR ν_{max} 3140, 2965, 2929, 2858, 1622, 1575, 1429, 1575, 1429, 1081, 830, 774 cm^{-1}; LCMS (ESI+): 333.2 ([M + H]$^+$, 100%), 355.3 ([M + Na]$^+$, 5%).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschemneuro.0c00677.

^{1}H and ^{13}C NMR spectra and LCMS trace for 5-fluoro-CBC (PDF)

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contributed to the acquisition and analysis of data. All authors contributed to drafting the manuscript or figures.

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Notes

No unexpected, new, or significant hazards or risks to report. The authors declare the following competing financial interest(s): Associate Professor Jonathon Arnold is Deputy Academic Director of the Lambert Initiative. He has served as an expert witness in various medicolegal cases involving cannabis and cannabinoids and served as a temporary advisor to the World Health Organization on their review of cannabis and cannabinoids. Iain McGregor is Academic Director of the Lambert Initiative for Cannabinoid Therapeutics. He has served as an expert witness in various medicolegal cases involving cannabis, has received honoraria from Janssen, is currently a consultant to Kinoxis Therapeutics, and has received research funding and fellowship support from the Lambert Initiative, NHMRC, and Australian Research Council. He currently sits on medical advisory board of BOD Australia and holds a variety of patents for non-cannabinoid therapeutics.

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■ ABBREVIATIONS

CBC, cannabichromene; 5-fluoro-CBC, 5-fluoro-cannabichromene; CBCA, cannabichromenic acid; CBCV, cannabichromevarin; CBCVA, cannabichromevarinic acid

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