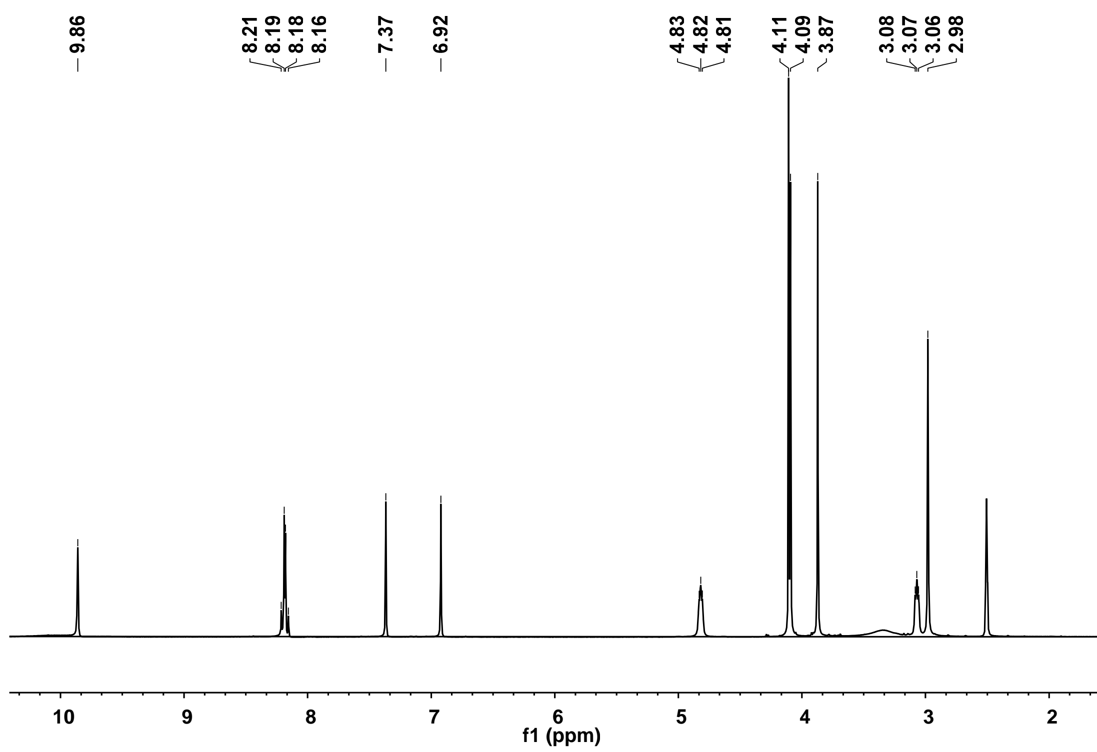
Structure Data

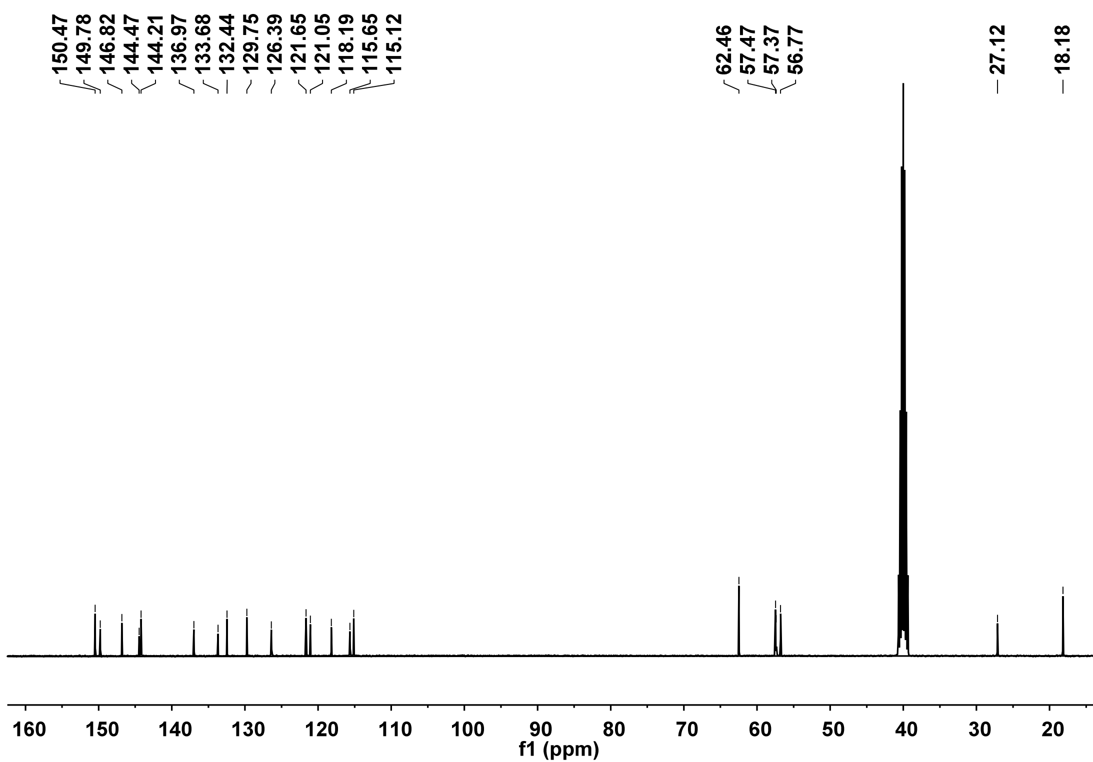
Data for purified DHCB: ^{1}H NMR (400 MHz, DMSO) δ 9.86 (s, 1H), 8.20 (d, J = 9.4 Hz, 1H), 8.17 (d, J = 9.4 Hz, 1H), 7.37 (s, 1H), 6.92 (s, 1H), 4.93 – 4.71 (m, 2H), 4.11 (s, 3H), 4.09 (s, 3H), 3.87 (s, 3H), 3.11 – 3.03 (m, 2H), 2.98 (s, 3H); 13C NMR (100 MHz, DMSO) δ 150.47, 149.78, 146.82, 144.47, 144.21, 136.97, 133.68, 132.44, 129.75, 126.39, 121.65, 121.05, 118.19, 115.65, 115.12, 62.46, 57.42, 56.77, 27.12, 18.18. HRMS (ESI+) Calc'd for $C_{21}H_{22}NO_4^+$ [M +] 352.1549, found 352.1543

NO.	δH	δC	HMBC
1	7.37 (s, 1H)	115.56	C-2, C-3, C-4a, C-14, C-14a
2		146.82	
3		118.19	
4	6.92 (s, 1H)	115.12	C-2, C-3, C-4a, C-5, C-14a
4a		132.44	
5	3.07 (t, 2H)	27.12	C-4, C-4a, C-6, C-14a
6	4.82 (t, 2H)	57.42	C-5, C-4a, C-8, C-14
8	9.86 (s, 1H)	144.21	C-6, C-8a, C-9, C-12a, C-14
8a		121.05	
9		144.47	
10		150.47	
11	8.17 (d, 1H)	121.65	C-9, C-10, C-12,C-12a
12	8.20 (d, 1H)	126.39	C-8a, C-11, C-12a, C-13
12a		133.68	
13		129.75	
13-Me	2.98 (s, 3H)	18.18	C-12a, C-13, C-14
14		136.97	
14a		149.78	
-OCH3	3.87 (s, 3H)	55.58	C-2
	4.11 (s, 3H)	61.16	C-9
	4.09 (s, 3H)	56.12	C-10

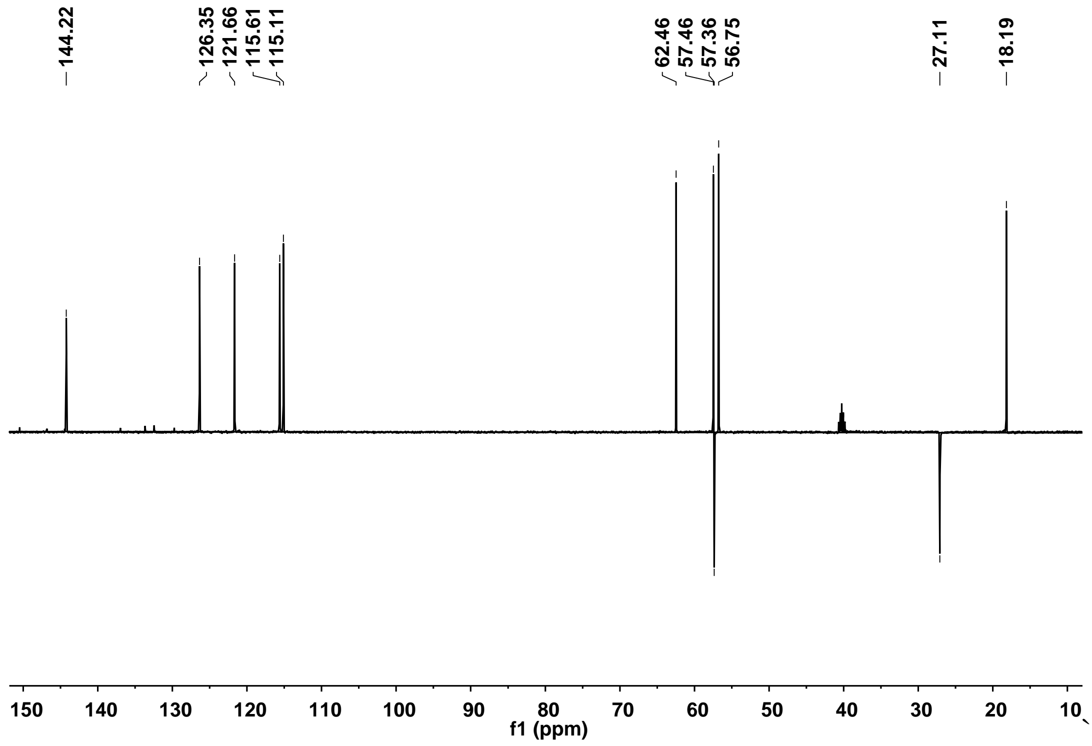
^{1}H spectra of purified DHCB

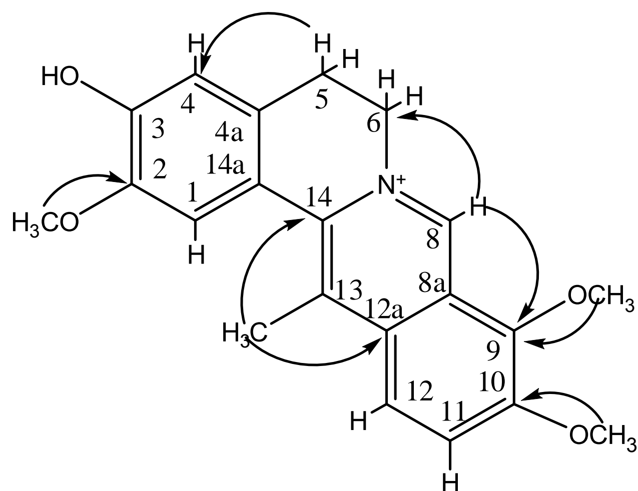


^{13}C spectra of purified DHCB

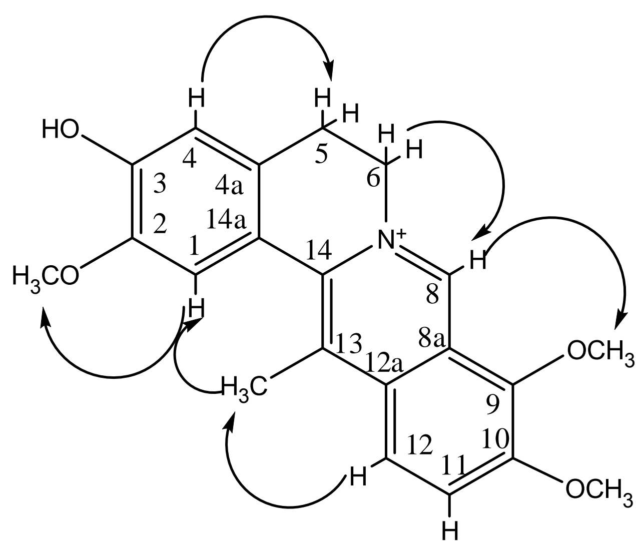


Dept135 spectral of purified DHCB



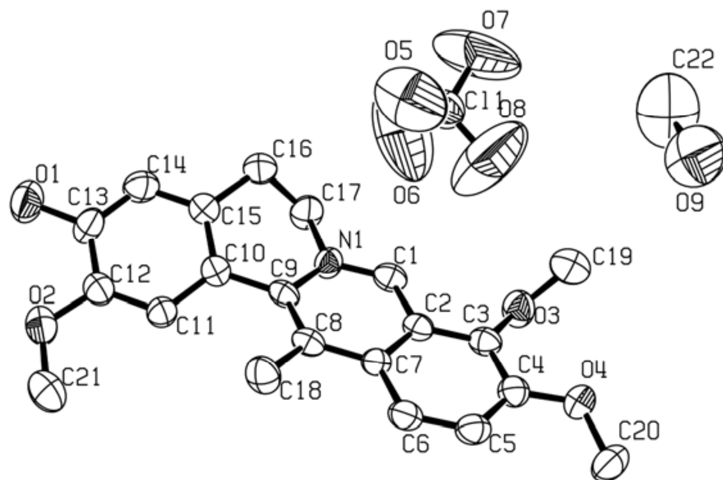


Key HMBC correlations of compound DHCB

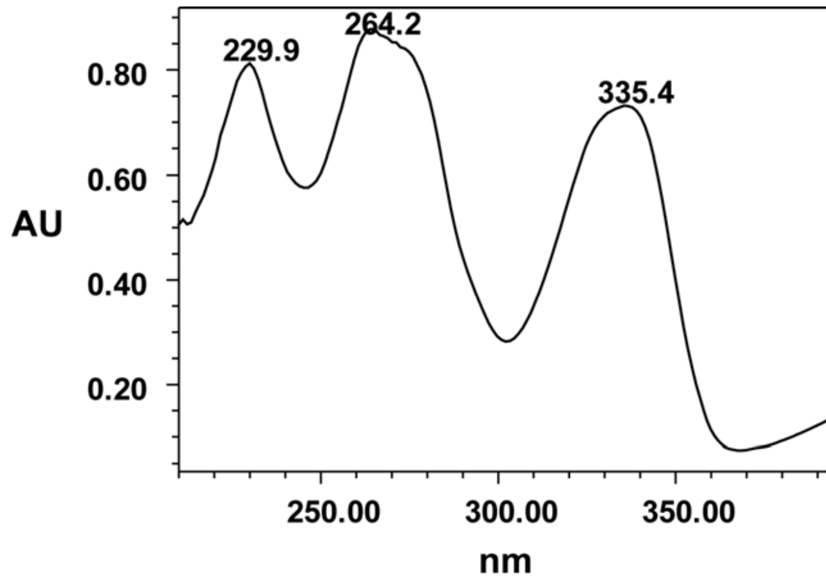


Key nosey correlations of compound DHCB

Crystal data for DHCB perchlorate: $C_{88}H_{104}Cl_{4}N_{4}O_{36}$, M= 1935.55, Monoclinic, space group Cmcm, a = 8.711(7) Å , b = 12.916(11) Å, c = 21.627(16) Å ,U=2272(3) Å 3, Z=1, Dc=1.415Mg m-3, μ(MoKR)=0.222 mm-1, T=293 K, 8224reflections collected. Refinement of 1789 reflections (298 parameters) with I>2σ(I) converged at final R1=0.0660, wR2=0.1593, and GOF = 1.048.

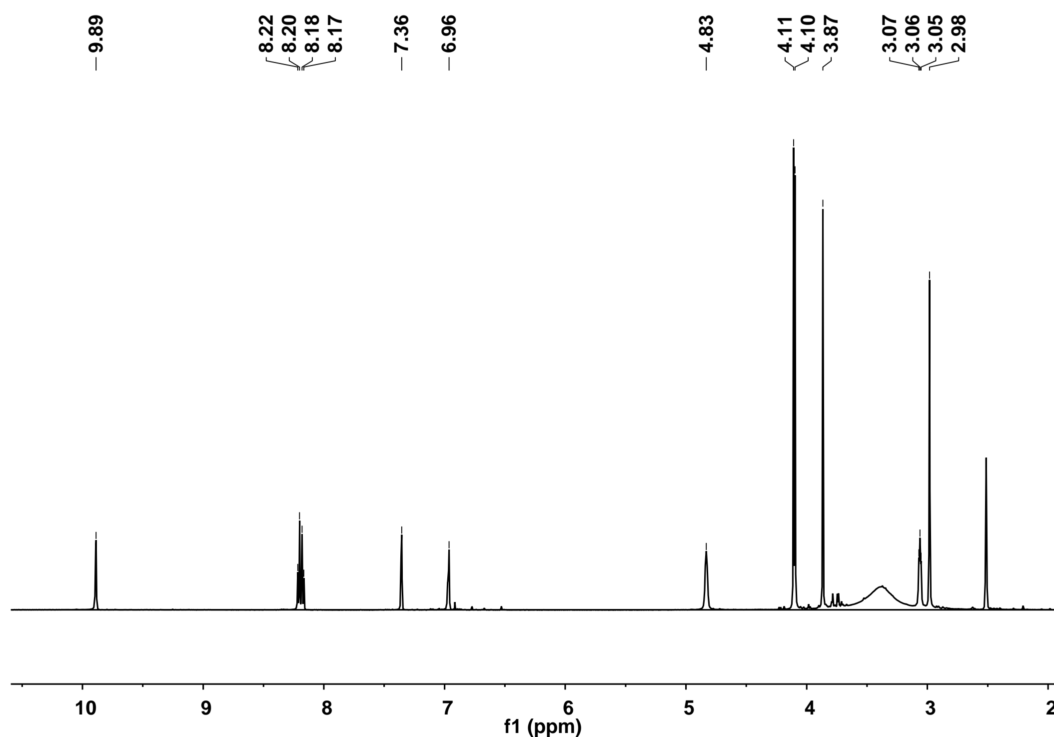


UV spectra of purified DHCB



Data for synthesized DHCB: ^{1}H NMR (600 MHz, DMSO) δ 9.89 (s, ^{1}H), 8.21 (d, J = 9.4 Hz, ^{1}H), 8.17 (d, J = 9.4 Hz, ^{1}H), 7.36 (s, ^{1}H), 6.96 (s, ^{1}H), 4.83 (s, ^{2}H), 4.11 (s, ^{3}H), 4.10 (s, ^{3}H), 3.87 (s, ^{3}H), 3.08 – 3.03 (m, ^{2}H), 2.98 (s, ^{3}H); ^{13}C NMR (150 MHz, DMSO) δ 150.43, 146.95, 144.44, 144.23, 144.20, 137.10, 133.70, 132.44, 129.57, 126.37, 121.67, 121.02, 117.95, 115.66, 115.20, 62.48, 57.48, 57.32, 56.74, 27.16, 18.22.

^{1}H spectra of synthesized DHCB



^{13}C spectra of purified DHCB

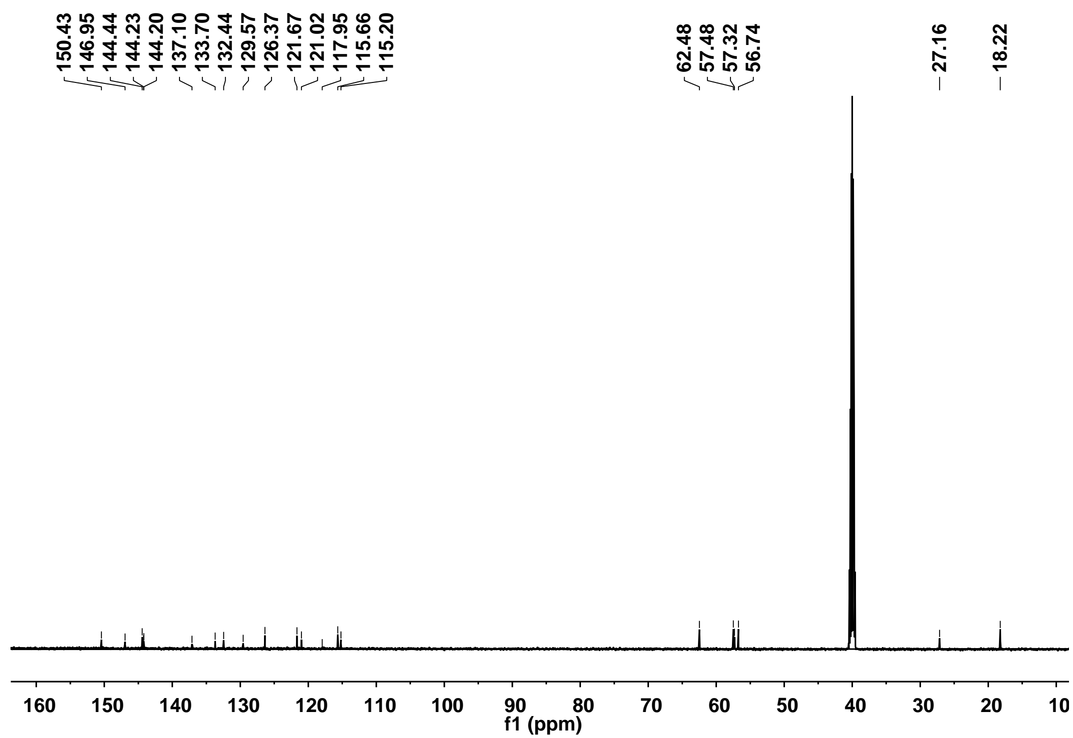
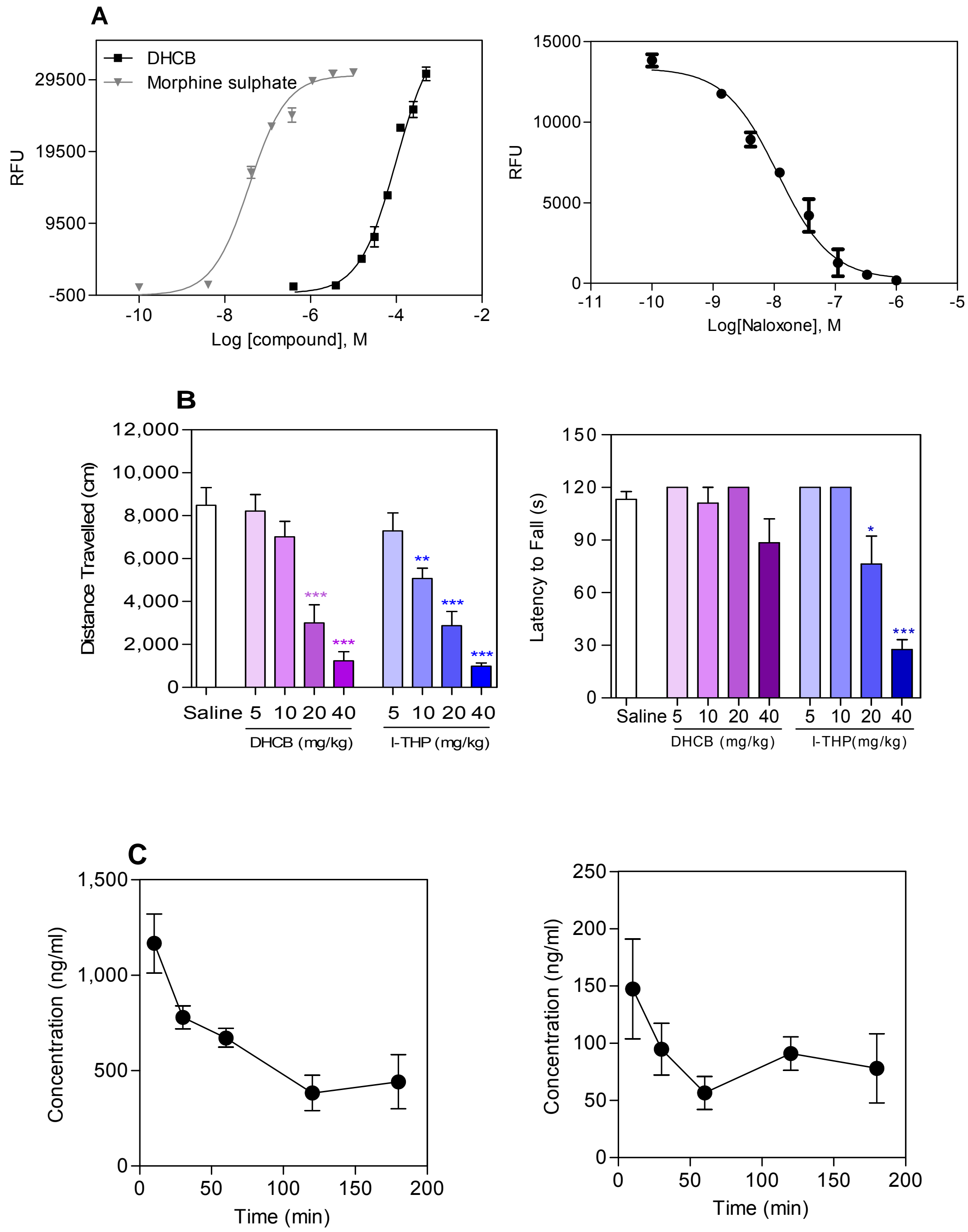
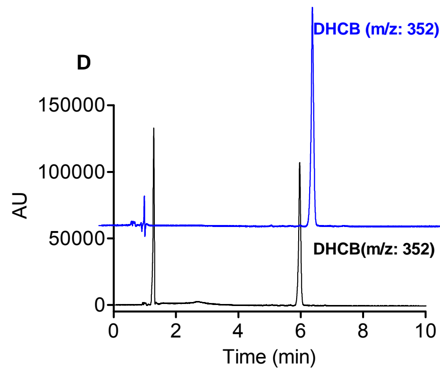
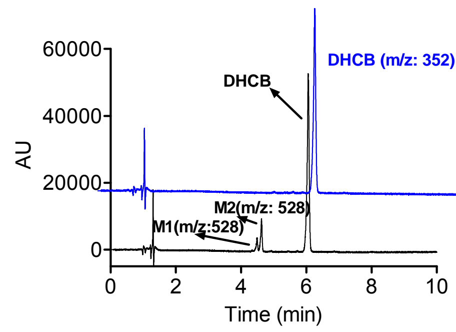


Figure S1







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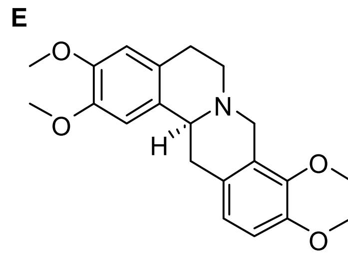

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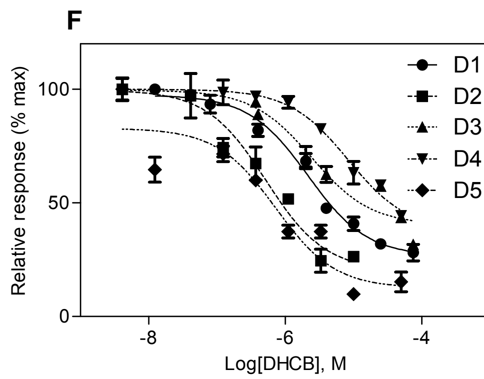
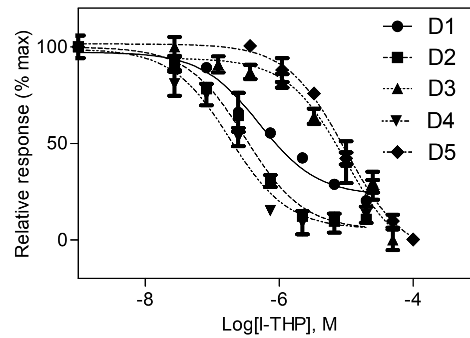
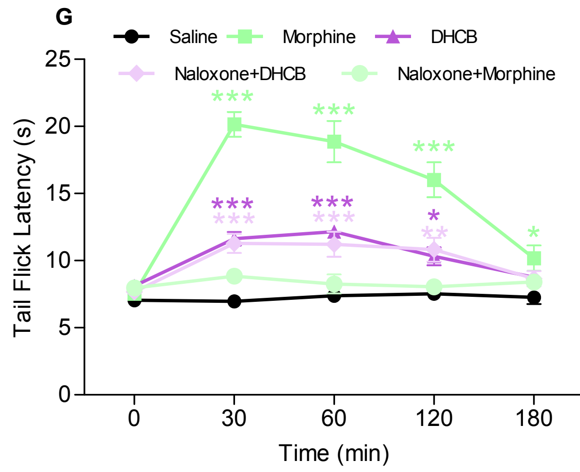
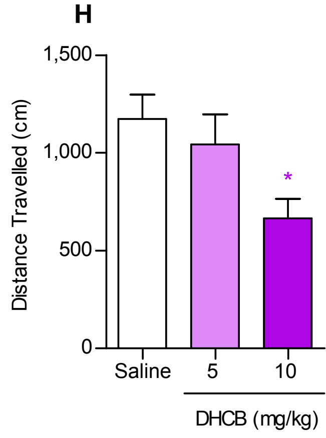





G
Saline
Morphine
DHCB
Naloxone+DHCB
Naloxone+Morphine
Tail Flick Latency (s)
Time (min)
0
30
60
120
180
25
20
15
10
5
0


H
Distance Travelled (cm)
1,500
1,000
500
0
Saline
5
10
DHCB (mg/kg)

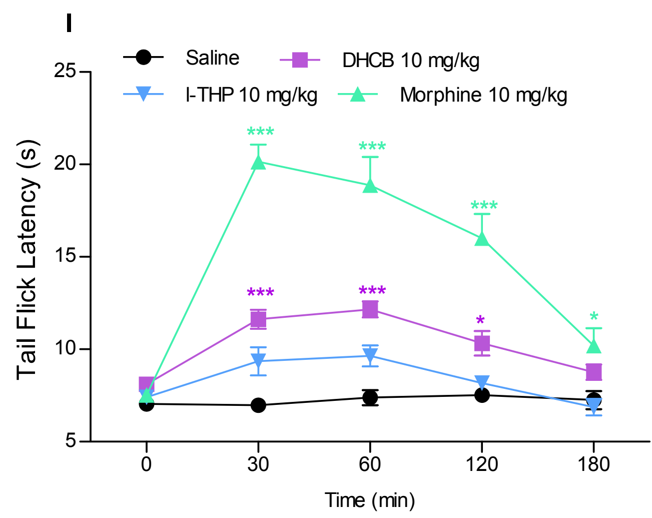

I
Saline
DHCB 10 mg/kg
l-THP 10 mg/kg
Morphine 10 mg/kg
Tail Flick Latency (s)
Time (min)
0
30
60
120
180
25
20
15
10
5

Figure S1. Antinociceptive effects of DHCB in the tail-flick assay and its mode of action.

(A) DHCB response in HEK293T cells expressing μ-opioid receptors monitored by Ca^{2+} mobilization. Left panel: DHCB dose-response curve. Morphine was used as a positive control. Right panel: Naloxone effects on the activity of DHCB (100 μM). The IC_{50} for naloxone is 7.977e-009 to 1.669e-008 M (95% confidence intervals). Error bars represent standard error of the mean of triplicate measurements for each point.

(B) The sedative effects of DHCB in CD1 mice. Left panel: 1-h locomotor activity test (n=10-13). One-way ANOVA revealed significant drug effect ($F_{8,90}$=18.55, $P < 0.0001$); Right panel: Rotarod performance test 30 min after drug injection (n=8). One-way ANOVA revealed significant drug effect ($F_{8,63}$=15.33, $P < 0.0001$). Dunnet's post hoc tests: drug vs. saline: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. In all cases, data are means ± S.E.M.

(C) Pharmacokinetic profile of DHCB (20 mg/kg, i.p.) in plasma (left panel) and brain (right panel) (n=4-5). Data are means ± S.E.M..

(D) Metabolism of DHCB *in vitro*. Left panel: Representative HPLC profiles of incubations of DHCB in HLM in the presence (black line) and absence (blue line) of an NADPH-regenerating system; Right panel: Representative HPLC profiles of the incubations of DHCB in HLM in the presence (black line) and absence (blue line) of UDPGA.

(E) Structure of l-THP.

(F) Inhibitory effects of DHCB (left panel) and l-THP (right panel) on dopamine induced Ca^{2+} mobilization in HEK293T cells expressing D1, D2, D3, D4, and D5 dopamine receptors, respectively. The individual dopamine receptors transfected cells were incubated with increasing concentrations of DHCB for 12 min before the addition of dopamine (250 nM of dopamine for D1, D2, D3, 25 nM for D4, and 50 nM for D5 receptor-expressing cells). Data from each dose curve were normalized to the maximal stimulation of each cell line (absence of DHCB). Error bars represent standard error of the mean of triplicate measurements for each point.

(G) Time-course of naloxone (1 mg/kg, i.p.) effect on DHCB response (10 mg/kg, i.p.) in the tail-flick assay (n=6-8). Naloxone was injected right before DHCB. CD1 mice were used in this

experiment. Two-way ANVOA revealed significant drug effect ($F_{4, 30}$=62.01, $P < 0.0001$), time effect ($F_{4,120}$=40.18, $P < 0.0001$) and drug × time interaction ($F_{16,120}$=10.72, $P < 0.0001$). Bonferroni post hoc tests: drug vs. saline: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

(H) Sedative effects of DHCB (5, 10 mg/kg, i.p.) in the mice used as wild-type control for the D2KO mice. Locomotor activity (n=7-21) was monitored for 1 h. One-Way ANOVA revealed significant drug dose effect ($F_{2,38}$=4.356, P=0.0198). Dunnet's post hoc tests: drug vs. saline: * $P < 0.05$. Data are means ± S.E.M.

(I) Time-course comparison of the antinociceptive responses obtained with DHCB (10 mg/kg, i.p.) and l-THP (10 mg/kg, i.p.) in the tail-flick assay (n=6-8). Two-way ANOVA revealed significant drug effect ($F_{3,23}$=116.15, $P < 0.0001$), time effect ($F_{4,92}$=41.87, $P < 0.0001$) and drug×time interaction ($F_{12,92}$=12.48, $P < 0.0001$). Bonferroni post hoc tests: drug vs. saline: ** $P < 0.01$, *** $P < 0.001$. Data are means ± S.E.M.

Figure S2

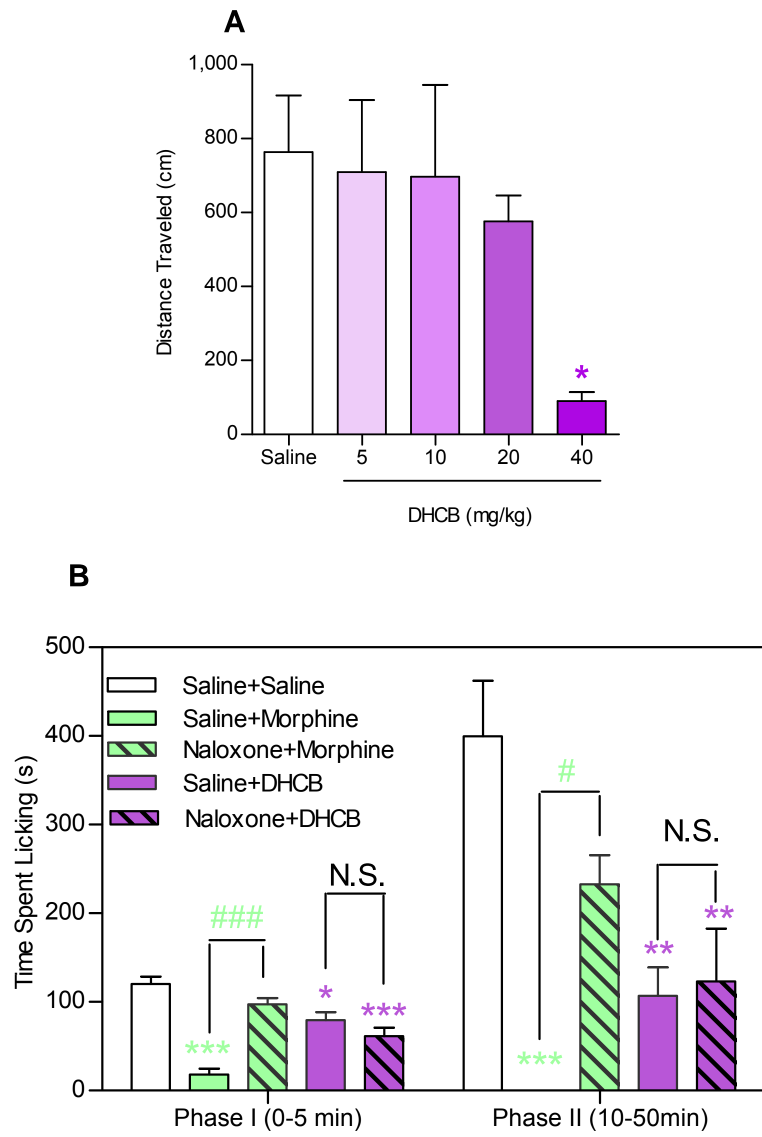


Figure S2. Antinociceptive effects of DHCB in tonic, persistent pain and neuropathic pain models.

(A) Sedative effect of DHCB in 129/sv mice (the wild type control for SNL mice). Locomotor activity was tested for 1 hr (n=9-17). One-way ANOVA revealed significant drug dose effect ($F_{4,50}$=2.68, P=0.042). Dunnet's post hoc tests: drug vs. saline: * $P < 0.05$. Data are means ± S.E.M.

(B) Naloxone (1 mg/kg, i.p.) effect on DHCB response (10 mg/kg, i.p.) in the formalin assay (n=7-10). Naloxone was injected right before DHCB. CD1 mice were used in this study. One-way ANOVA revealed significant drug effect both in phase I ($F_{4,38}$=18.55, $P < 0.0001$) and phase II ($F_{4,38}$=9.779, $P < 0.0001$), Bonferroni post hoc tests: drug vs. saline: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; morphine vs. morphine+naloxone: # $P < 0.05$, ### $P < 0.001$; N.S., not significant, $P > 0.05$. Data are means ± S.E.M.

Supplementary Experimental Procedures

Plant material

The tuber of *C. yanhusuo* was collected from Dongyang County (Zhejiang, China) and authenticated by Institute of Medication, Xiyuan Hospital of China Academy of Traditional Chinese Medicine. The tuber was first processed with vinegar as used in the traditional way to enhance the analgesic effect. Other nine TCMs including Commiphora myrrha (Nees) Engl., Macleaya cordata (Willd.) R.Br., Stephania japonica (Thunb.) Miers, Stephania tetrandra S. Moore, Huperzinaserrata (Thumb.)Trev, Anisodus tanguticus (Maxinowicz) Pascher, Nandina domestica, Carthamus tinctorius L, Lonicera japonica Thunb were collected from their mainly distributed locations in China and authenticated by Institute of Medication, Xiyuan Hospital of Chinese Academy of Traditional Chinese Medicine.

Plant extraction and fractions library

The extraction of *C. yanhusuo* was performed by Mai-Di Hai Pharmacy (China). The procedures were as follows: 10 kg of herb was ground into powder and decocted in 100 L of water at 100 °C for 120 min. Then the residue was collected and redecocted in 100 L of water at 100 °C for 90 min. The decoctions were pooled together and dried by spray drying to yield 0.6 kg water extract. Then 0.15 kg of the extract was dissolved in 1.5 L of water/ethanol 30:70 (v/v) and the solution was extracted with 0.75 L ethyl acetate three times. Residual was then extracted with 0.75 L n-butanol three times and the fraction of n-butanol was collected and dried with a rotary evaporator at 60 °C to yield 45 g of extract. A 1.0-g portion of the n-butanol extract was dissolved in 5 ml methanol/water/formic acid (50:50:0.1, v/v/v) and filtered through 0.22 μm membranes to make the crude sample with a concentration of about 200 mg/ml. The fractions library for the other TCMs were established as described earlier [S1].

Animal studies

Analgesic assays

Tail-flick assay. This method was originally described by D' Amour et al. In brief, the acute pain response was measured using an electronically controlled tail-flick analgesimeter (UGO basile biological research apparatus, 7360 Tail Flick) that integrated both a thermal nociceptive stimulus and an automated response timer. A thermal stimulus (focused light from a 20W infrared bulb as the heat source) was directed on the tips of the mouse tails. The time from onset of stimulation to a rapid withdrawal of their tails from the heat source was recorded as tail flick latency. A maximum of 22 seconds was set as a cut off time to prevent tissue damage. After three days' baseline measures, mice were injected (5 ml/kg, i.p.) with saline, morphine (10 mg/kg), or DHCB

(5-40 mg/kg) or l-THP (10 mg/kg) and tail-flick latency was measured 30, 60, 120 and 180 minutes after drug injection. For some of the experiments, naloxone (1 mg/kg) or saline was injected (2.5 ml/kg, i.p.) right before the drug administration. In this case (naloxone presence), tail-flick latency was measured 30 min after drug injection. SKF-38393 (1 mg/kg) or quinpirole (0.5 mg/kg) was injected (5 ml/kg, i.p.) 30 min prior to DHCB injection. In this case (SKF-38393 or quinpirole presence), tail-flick latency was measured 30 min and 60 min after DHCB injection. The experimenter was blind to all treatment conditions.

Formalin Paw Test: The formalin test in mice was performed according to the methods described by Hunskaar et al.. In brief, mice were placed individually in a 4 liters glass beaker and were allowed to acclimate for 30 min before the test. Saline, morphine (10 mg/kg), or DHCB (5, 10 mg/kg) or l-THP (10 mg/kg) was injected (5 ml/kg, i.p.) 15 minutes prior to formalin injection. Twenty five μl of 5 % formalin solution was injected into the dorsal surface of the right hind paw using a 50 μl Hamilton syringe with a 30 gauge needle. Immediately after formalin injection, mice were placed individually in the beaker and a mirror was arranged in a 45 °C angle under the beaker to allow clear observation of the paws of the animals. The nociceptive behavior (paw licking) was observed continuously for 50 minutes. The time animals spent on licking the injected paw during the first phase (0-5 min) and second phase (10-50 min) were recorded at 5 min - internals. Additionally, the effects of DHCB in the presence of the opioid receptor antagonist naloxone were evaluated using the formalin test. For some of the experiments, naloxone (1 mg/kg) or saline was injected (2.5 ml/kg, i.p.) right before the drug administration. The experimenter was blind to all treatment conditions.

Neuropathic pain assay in spinal nerve ligation (SNL) model

SNL: Unilateral SNL injury was performed in male adult mice as described [S2]. Briefly, the left L4 spinal nerve [S3] was exposed in an isoflurane anesthetized mouse and ligated with a silk suture between DRG and the conjunction of sciatic nerve. Sham operations were performed in the same way except that spinal nerves were not ligated.

Drug treatment: Mice were injected (5 ml/kg, i.p.) with DHCB (10 mg/kg) about two weeks post SNL when all injured mice have developed hindpaw mechanical and thermal hypersensitivities on the injured side.

Behavioral tests: _Von Frey filaments test:_ Mouse 50% paw withdrawal thresholds (PWT) to calibrated von Frey filament (Stoelting, Wood Dale, IL) stimulation were assessed in both hindpaws with a modified up-down method of Dixon [S4] before and after SNL (0, 30, 60, 120, 180 and 240 min after drug treatment) as described previously. Briefly, mice were acclimated for

1 h on a mesh surface of the test apparatus, a series of von Frey filaments (buckling force between 0.04 and 2.0 gm), starting with a 0.4 gm one, was applied to hindpaw plantar surface. A positive response of paw lifting within 5 sec led to the use of the next weaker filament. Absence of paw lifting after 5 sec was considered a negative response and led to the use of the next filament with increasing force. Scores of six measurements, starting from the one prior to the first positive response, were used to calculate the 50% PWT except that a score of 0.01 gm was assigned to four consecutive positive responses or a score of 3.0 gm was assigned to three consecutive negative responses.

<u>*Hot box assay:*</u> Mouse hindpaw withdrawal latencies (PWL) to a thermal stimulus were measured in a modified Hargreaves-type hot box [S5]. Briefly, mice were acclimated for at least 30 min within individual boxes on the hot box glass surface maintained at 30 °C. A radiant light source under the glass surface was aligned to the hindpaw planter surface. A timer was activated when the light source was turned on and stopped when a paw withdrawal from the light source was detected by motion sensors or at 20 sec of light stimulation to prevent thermal injury.

Sedative effects measurements

<u>*Spontaneous locomotor activity study:*</u> The sedative effects of the drugs were evaluated 30 min after drug administration by measuring the spontaneous locomotor activity for 1 h as previously reported [S6]. Because of the possible sedative effect of the test drugs, the habituation step was skipped. 30 min after mice were injected (5 ml/kg, i.p.) with saline or DHCB (5-40 mg/kg) or l-THP (5-40 mg/kg), they were placed into an open field test chamber (40 x 40 cm, med associates, inc.). Horizontal activities were measured by the infrared beam arrays. The total distance animals travelled for 1 h was recorded, analyzed and calculated by Activity Monitor 5 software (Med associates, inc.) and used to evaluate the effects of drugs on locomotion.

<u>*Rotarod test:*</u> The rotarod test was performed according to the methods originally described by Dunham et al. with some modification [S7]. In brief, mice were trained for three consecutive days, two trails per day (9:00 am and 3:00 pm), to maintain their position on a rotarod apparatus (Columbus Instruments) with a diameter of 3 cm, at a fixed speed of 11 rpm. Each trail consisted of walking on the rotarod for a maximum of 120 sec. Only animals that could easily remain on the rod for at least 60 sec after training were used. On the fourth day of the test, mice were injected (5 ml/kg, i.p.) with saline, DHCB (5-40 mg/kg) and l-THP (5- 40 mg/kg) 30 min before testing. Mice were then placed on the rotarod 30 min after drug administration and the latency to fall (sec) was recorded. The cut-off time was set at 120 sec.

<u>**Study the DHCB Pharmacokinetics**</u>

Mice were injected with a single dose of DHCB (20 mg/kg, 5 ml/kg, i.p.). Blood was collected at 10, 30, 60, 120, and 180 minutes as indicated, into tubes containing EDTA. Plasma was collected after centrifugation (3500 g). Brains were also excised at those time points after perfusion with saline. The plasma and brain were mixed with acetonitrile; brain samples were sonicated with a probe tip sonicator for 20 min to break up the tissue. Drug levels in brain and plasma were measured by API2000 LC-MS/MS (AB Sciex, Foster City, CA, USA) and calculated by comparing internal standards prepared in plasma and brain matrix.

Study the DHCB metabolism

The potential NADPH-dependent (phase I metabolism) and UGT-mediated (phase II metabolism) metabolism of DHCB was studied by incubating with human liver microsomes (HLM) *in vitro* as previously described [S8]. UFLC-DAD (Shimadzu, Japan) and UFLC-ESI-MS (Shimadzu, Japan) were used for the analysis.

Supplemental references

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