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Pharmacological characterization of the neurotrophic sesquiterpene jiadifenolide reveals a non-convulsant signature and potential for progression in neurodegenerative disease studies

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Abstract

The ‘neurotrophic sesquiterpenes’ refer to a group of molecules derived from the *Illicium* genus of flowering plant. They display neurotrophic effects in cultured neuron preparations and have been suggested to be cognitive enhancers and potential therapeutics for neurodegenerative disorders and dementias. Recent synthetic advances generated sufficient quantities of jiadifenolide for in vivo investigation into its biological effects. Jiadifenolide did not induce convulsions in mice nor did it enhance or diminish convulsions induced by pentylenetetrazole. Other negative allosteric modulators of GABA_A receptors, picrotoxin, tetramethylenedisulfotetramine (TETS), and bilobalide all induced convulsions. Either i.p. or i.c.v. dosing generated mM plasma and brain levels of jiadifenolide but only small effects on locomotion of mice. However, jiadifenolide decreased *d*-amphetamine-induced hyperlocomotion in mice, an antipsychotic-like drug effect. Jiadifenolide did not significantly alter body temperature or behavior in the forced-swim test in mice. Molecular simulation data suggested a potential site in the pore/M2 helix region that is at an overlapping, yet lower position than those observed for other ‘cage convulsant’ compounds such

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as TETS and picrotoxin. We hypothesize that a position nearer to the entrance of the pore channel may allow for easier displacement of jiadifenolide from its blocking location leading to lower potency and lower side-effect liability. Like jiadifenolide, memantine (Namenda), one of the few drugs used in the symptomatic treatment of dementias, occupies a unique site on the NMDA receptor complex that creates low binding affinity that is associated with its reduced side-effect profile. Given the potential therapeutic applications of jiadifenolide and its relatively inert effects on overt behavior, the possibility of clinical utility for jiadifenolide and related compounds becomes intriguing.

Abstract

1. Introduction.

The ‘neurotrophic sesquiterpenes’ refer to a group of molecules derived from the *Illicium* genus of flowering plant. Neurotrophic actions of jiadifenolide have been reported in cultured rat [1] and human [2] cortical neurons. Neurotrophic actions of compounds suggest potential therapeutic utility in neurodegenerative disease states including major depressive disorder where neurotrophic phenomenon have been implicated in therapeutic relief [3]. For example, two studies with picrotoxin have shown that picrotoxin, in non-convulsant doses can help restore cognitive function in Ts65Dn mice that are used to model aspects of Downs syndrome [4]. Related effects of picrotoxin have been reported in Alzheimer’s disease rodent models such as the APP/PS1 mouse model [5].

There has been little work done on the pharmacological characterization of these compounds due to the complexity of chemical synthesis. There have been even fewer studies characterizing the effects of these molecules on biological systems *in vivo*. In one study over 20 years ago, three sesquiterpenes were isolated from star anise [6]. These compounds produced hypothermia in mice and at somewhat higher doses (3 mg/kg, *p.o.*), convulsions and lethality were observed (Nakamura et al., 1996). In the same study, efficacy against

methamphetamine-enhanced locomotor activity and analgesia were also reported at low doses.

Lu et al. [7] and Ohtawa et al. [8] (2017) provided efficient synthetic routes that have enabled quantities of material for in vivo investigation. We thus set out to characterize the in vivo pharmacology of jiadifenolide (Fig. 1) in rodents. Based upon the well-known convulsive pattern induced by the structurally similar compound picrotoxin [9], and the electrophysiological data showing commonalities in the properties of tashironin and picrotoxinin [8], we initially hypothesized that jiadifenolide would be convulsant. Since this effect was not engendered, we then attempted to uncover a signature of jiadifenolide in vivo by exploring other behavioral outcomes. The lack of marked effects of jiadifenolide in vivo in comparison to compounds with some structural and mechanistic overlap (picrotoxin, tetramethylenedisulfotetramine (TETS), and bilobalide) led us to hypothesize that jiadifenolide binds within a novel pocket from other caged convulsants. Molecular simulation data suggested a potentially novel site of interaction that could account for the lower potency and reduced side-effect liability of jiadifenolide. This mechanism of action might prove therapeutically beneficial.

2. Materials and Methods

2.1. Compounds.

Jiadifenolide and tashironin were synthesized by us [7,8]. Other compounds were obtained from commercial sources: pentylenetetrazole (PTZ), picrotoxin and tetramethylenedisulfotetramine (TETS) were from Sigma-Aldrich (St. Louis, MO, USA), and bilobalide was purchased from AdipoGen Life Sciences, Adipogen Corporation (San Diego, CA, USA).

Picrotoxin and PTZ were dissolved in 0.9% NaCl. TETS was diluted from the manufacturer stock solution of 100ug/ml as needed in 0.9% NaCl. Jiadifenolide and bilobalide were suspended in 1% hydroxyethylcellulose/0.25% Tween-80/0.05% Dow antifoam in water. Doses and routes of administration of the molecules were determined from the experimental literature.

2.2. Rodent Assays.

All studies were performed in accordance with guidelines of the National Institutes of Health and by local animal care and use committees. The local animal care and use committee and veterinary staff provided direct oversight of the animals by inspections, protocol reviews, laboratory site visits, and animal health monitoring.

Animals were housed separately by species in a quiet, ventilated-, temperature- and humidity-controlled vivarium that met AALAC accreditation. Lighting was controlled with a 12 h light-dark cycle (lights on at 6 am). Food and water were available to the animals at all times when the animals were in their home cages. They were maintained in the colony room for at least 3 days before testing. Animals were moved to a quiet room 1 hour prior to the start of the test.

Male, CF-1 (20–28 g) mice (Envigo, Indianapolis, IN) or Male, NIH, Swiss mice (28–32g) (Harlan Sprague-Dawley, Indianapolis, IN) were used. Animals were transferred from the vivarium to the testing area in their home cages and allowed to adapt to the new environment for at least one hour before testing.

Drug-induced convulsions.—Male, CF1 mice were used in these experiments with the minimal number needed to enable statistically-significant detection of drug effects. Mice were placed individually into small, clear plastic cages and allowed to explore and acclimate for 30 minutes. Then each mouse was dosed with jiadifenolide, TETS, PTZ, or bilobalide either alone or in combination with PTZ. Visual observations began after dosing by a trained observer. In addition to recording behavioral changes, the occurrence of convulsions was also recorded with clonic convulsions defined as repetitive movement of fore- and hindlimbs during which the mouse demonstrated loss of righting. Tonic convulsions were defined as loss of righting along with tonic hindlimb extension. The percentage of animals exhibiting convulsions was analyzed by Fisher's Exact probability test with the error rate being set at 0.05.

Locomotor activity.—Male, CF-1 mice (Envigo, Indianapolis, IN) were used. Animals were transferred from the vivarium to the testing area in their home cages and allowed to adapt to the new environment for at least one hour before testing. Locomotor activity was measured with a 20 station photobeam activity system (San Diego Instruments, San Diego, CA, USA) with seven photocells per station. Locomotor activity was recorded as the number of ambulations, where ambulation was defined as the breaking of adjacent photobeams. Animals were placed individually into polypropylene cages (40.6 × 20.3 × 15.2 cm, no bedding) immediately post injection. In the case of experiments with *d*-amphetamine, jiadifenolide (150 mg/kg, i.p.) or picrotoxin (1 mg/kg, s.c.) was dosed 30 min prior to *d*-amphetamine and the mice placed into the locomotor arenas post *d*-amphetamine or vehicle injections. Locomotion was monitored for up to 120 min post dosing. Dose-effect and time-course data were analyzed by 2-way ANOVA followed by Dunnett's test with $p < 0.05$ considered significant.

Forced-swim assay.—This assay that detects antidepressant drugs [10] was conducted as described [11]. Male, NIH Swiss mice were dosed with jiadifenolide i.c.v. and tested in the assay 20 min post dosing. The mice were scored by a trained observer for the amount of time the mice remained immobile (making only those movements necessary to keep their heads above water). The data were analyzed by ANOVA followed by post-hoc Dunnett's multiple comparison test at $\alpha = 0.05$.

Body temperature.—Male CF-1 mice were removed from their home cage and dosed with jiadifenolide (150 mg/kg, i.p.) and placed in a holding cage. Thirty min and 60 min post dosing, body temperature was taken using a rectal probe (Physitemp Instruments Inc. (Clifton, NJ, USA) Model BAT-12, Probe RET-3 inserted 1.8 cm. Temperature was recorded in degrees Celsius.

Brain and Plasma Exposures.—Three mice from each dose group (0, 30, and 150 mg/kg) from the locomotor activity experiment were sacrificed at 120 min post dosing and

plasma and whole brain were extracted. Brain samples were weighed and a 3-fold volume of water/methanol (4:1, v/v) was added prior to homogenization with an ultrasonic tissue disrupter. Control (naïve) brain tissue was also homogenized to generate control homogenate for preparation of calibration standards. A 25 μ L aliquot of each study sample, appropriate calibration standard, and control plasma or brain homogenate were mixed with 180 μ L of acetonitrile/methanol (1:1, v/v) containing internal standard. After mixing, the samples were centrifuged to pellet the precipitated proteins, and 10 μ L aliquots of the resulting supernatant were analyzed by LC-MS/MS using a Sciex API 4000 triple quadrupole mass spectrometer (Applied Biosystems/MDS; Foster City, CA) equipped with a TurboIonSpray interface, and operated in negative ion mode. The analytes were chromatographically separated using a Betasil C18 5 μ m 20 $\times$ 2.1mm Javelin (Thermo Electron Corp, Waltham, MA). The pumps were Shimadzu LC-10ADvp units with a SCL-10A controller (Kyoto, Japan), and a Gilson 215 liquid handler (Middleton, WI) was used as the autosampler. The flow rate was 1.5 mL/min from 0 to 0.49 min, and increased to 2.5 mL/min from 0.50 to 0.72 min. For one of the compounds, the mobile phase system was composed of Water/1M NH₄HCO₃ (2000:1 v/v) (Mobile Phase A) and acetonitrile (Mobile Phase B). The gradient profile over the 0.72 min was 10% B at 0 min to 0.20 min, linear gradient from 10% B at 0.20 to 40% B at 0.4 min, and 98% at 0.41 to 0.72 min. The selected reaction monitoring (M-H) transition m/z was 309.0 > 247.0.

2.3. Electrophysiological characterization.

Electrophysiology methods utilized the Ion Works Barracuda (IWB) system. Unless otherwise specified, all cell culture reagents were obtained from Thermo Fisher Scientific (Waltham, MA), and all other reagents were obtained from Sigma-Aldrich (St Louis, MO). Compound serial dilutions were prepared in dimethyl sulfoxide (DMSO) and diluted into external recording solution prior to the experiment. Final DMSO concentration was 0.3%.

HEK293 cells stably expressing hGABAAR ($\alpha 1\beta 3\gamma 2$) were obtained from ChanTest (Cleveland, OH). Cells were cultured at 37 °C in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 1% non-essential amino acids, G418 (200 μ g/ml), hygromycin (100 μ g/ml), zeocin (50 μ g/ml) and puromycin (0.1 μ g/ml). For IWB experiments, cells were grown in T-150 flasks (Corning) to 80–90% confluency, rinsed twice with D-PBS without calcium and magnesium and dissociated by incubating in 3 mL of TrypLE™ (Thermo Fisher Scientific, Waltham, MA) for 5 min. Cells were then re-suspended in 10 mL of media, gently triturated, and centrifuged for 5 min at 1000 RPM. The supernatant was aspirated and the cells resuspended in 5 mL HBSS-based external solution (see below). During IWB recordings, cells were supplied and seals established in an external solution consisting of Hanks Balanced Salt Solution (HBSS) supplemented with 20 mM HEPES with pH adjusted to 7.4 using NaOH. The internal solution used was (in mM): 90 K-Gluconate; 40 KCl, 3.2 MgCl₂; 3.2 EGTA and 5 HEPES; with pH adjusted to 7.2 using KOH. The membrane perforating agent amphotericin B was prepared as a 28 mg/mL stock solution in DMSO on the day of the experiment and added to the internal solution at a concentration of 0.1 mg/mL.

Electrophysiological recordings were made as follows: The IWB instrument was primed with intracellular and extracellular solutions and 7 μ L of cells (5M/mL) added to each well. Recordings were performed in population patch clamp (PPC) mode, and the whole cell recording configuration was established by 8 min incubation with amphotericin B. The holding voltage was set to -80 mV and sampling frequency was 1 kHz. Test compound was added during first compound addition. After a three-minute incubation with compound, GABA currents were evoked with co-administration of GABA at approximately an EC_{90} concentration together with test compound. Data acquisition, leak subtraction and initial analysis of peak currents during each test pulse were performed using IWB software (version 2.0.2; Molecular Devices Corporation, Union City, CA). Compounds were tested at 10 concentrations in three to eleven replicates. Mean and standard error of the normalized peak current amplitudes were fit to the Hill equation using GraphPad Prism 6.02 software (GraphPad Software, San Diego, CA).

2.4. Radioligand Binding.

To determine other potential sites of interaction of jiadifenolide, radioligand displacement studies were generously provided by the National Institute of Mental Health's Psychoactive Drug Screening Program. Methods are available at <https://pdspdb.unc.edu/pdspWeb> where these data are also available. Single point screening was conducted at a concentration of 10 μ M.

2.5. Molecular Simulations.

The ligand-binding searches were conducted using a combination of molecular dynamics (MD) simulations and docking protocols. The GABA_A receptor (GABA_AR) $\alpha_1\beta_6\delta$ homology model was built with a GABA_AR β_3 homopentamer template (PDB ID code: 4COF) [12] using previously published protocols [13,14]. Jiadifenolide was parameterized using the ATB server [15]. The GABA_AR $\alpha_1\beta_6\delta$ model was inserted into a preformed POPC (palmitoyl-oleoyl phosphatidylcholine) lipid bilayer. The system was solvated and had counter-ions added to neutralize the charge. Simulation systems comprised a hydrated lipid bilayer with 272 POPC lipids, the membrane-embedded GABA_AR model, $\sim 37,000$ water molecules, 150 mM NaCl. The entire system was energy minimized using steepest descents. The system was then equilibrated for 200 ns while the protein backbone was restrained to allow for relaxation of both the membrane and the amino acid sidechains.

Following equilibration of the protein-membrane system, 50 jiadifenolide molecules were randomly added into the solvent of the system. In a similar approach to previous methodologies used to identify binding pockets on ligand-gated ion channels [16,17], three separate repeat simulations of 500 ns each were run to allow jiadifenolide to sample the surface of the GABA_AR model. The accumulated density of jiadifenolide over the final 200ns of each simulation was used for further analysis.

The MD simulations of the GABA_AR $\alpha_1\beta_6\delta$ protein were carried out using GROMACS v5 [18]. MD simulations were run using the CHARMM36 force field [19] as implemented in GROMACS [20]. The water model used was TIP3P [21] with CHARMM modifications [22]. Hydrogen atoms were constrained using the LINCS algorithm [23]. Lennard-Jones

interactions were calculated using a cutoff that is smoothed to zero between 0.8 and 1.2 nm. Electrostatic interactions beyond 1.2 nm were calculated using the smooth particle-mesh Ewald (PME) method [24,25]. The simulation were run at a pressure of 1 bar using semi-isotropic pressure coupling with the Parrinello-Rahman barostat [26] with a coupling constants of 5 ps and at a temperature of 303 K using a Nosé-Hoover thermostat [27] with a coupling constant of 0.5 ps. The integration time step was 2 fs.

Ligand docking was carried out using the methods and protocols as described [28,29]. Briefly, following a VinaLC docking calculation, molecular mechanics/generalized Born surface area (MM/GBSA) rescoring was calculated for the top ten docked ligand poses. The more accurate solvation terms and energy function of the MM/GBSA method allow better ranking of the poses.

3. Results

3.1. Convulsant effects.

Doses of from 10–150 mg/kg, i.p. failed to show any striking overt effects in male, CF-1 mice. There were no convulsions (Fig. 2). Dosing i.c.v. at either 1 or 10 μ M also did not engender convulsant activity. It was noted however, that compared to vehicle-treated mice, the mice treated with jiadifenolide (i.c.v.) were more active, moving about the cage and exploring while the vehicle treated mice were generally huddled together resting (Fig. 2).

As there were no convulsions, we then evaluated the possibility that jiadifenolide might either exacerbate or protect against convulsions induced by pentelenetrazole (PTZ). No enhancement or blockade by jiadifenolide was observed when studied against either 45 or 60 mg/kg PTZ (Table 1).

Given the lack of convulsant activity of jiadifenolide, we then explored the behavioral and convulsant effects of molecules of generally similar structures (TETS, picrotoxin, and bilobalide). The known convulsant agents, TETS and picrotoxin, were convulsant in our hands (Fig. 2).

In contrast, bilobalide (30 mg/kg, p.o.), did not induce convulsions. However, when given in combinations with low convulsant doses of PTZ, bilobalide significantly increased the number of mice exhibiting clonic convulsions (Table 2). In higher doses of bilobalide alone (100 and 300 mg/kg), bilobalide also produced convulsions (Fig. 2).

3.2. Locomotor activity.

Quantification of movement of the mice at 30 and 150 mg/kg (i.p.) likewise showed only small behavioral modifications (~35%) that were not dose-dependent $p=0.35$) (Fig. 3A). The effects of dose ($F_{2,252} = 6.78$, $p<0.01$) and time ($F_{11,252} = 25.5$, $p<0.0001$) were significant as was the dose $\times$ time interaction ($F_{22,252} = 2.04$, $p<0.05$). Post-hoc analysis by Tukey's test revealed that effects of vehicle were significantly different than those produced by 30 mg/kg ($p<0.01$) and showed a trend for significance at 150 mg/kg ($p=0.06$).

In order to determine the degree to which jiadifenolide exposed the brain and plasma of mice, we took biological samples from three mice in the vehicle, 30 and 150 mg/kg groups and evaluated the concentrations of jiadifenolide in plasma and brain at 120 min post-dosing. Table 3 shows that the exposures of jiadifenolide in plasma and brain of mice at this time point were dose-dependent and reached mM concentrations in both compartments. The [brain]/[plasma] was ~5 at 30 μ M jiadifenolide and ~2 at 150 μ M jiadifenolide (Table 3). We also estimated the unbound drug concentrations in each biological compartment (Table 3).

Comparable to intraperitoneally-dosed jiadifenolide, i.c.v. administration (10 or 30 μ M) also produced only small effects on locomotion of mice (Fig. 3B). In that experiment, effects of dose ($F_{2,378} = 7.1$, $p < 0.001$) and time ($F_{17,378} = 9.37$, $p < 0.0001$) were significant with a non-significant; dose $\times$ time interaction ($F_{34,378} = 1.17$, $p = 0.24$). Post-hoc analysis by Tukey's test revealed that effects of vehicle were significantly different than those produced by 10 μ M ($p < 0.05$) but not that of 30 μ M ($p = 0.58$). As with the 10 μ M dose, 30 μ M jiadifenolide (i.c.v.) also did not engender convulsions.

3.3. Amphetamine-induced hyperlocomotion.

At 2 mg/kg (s.c.), *d*-amphetamine stimulated locomotion of mice (Fig. 4). This hyperlocomotion was significantly attenuated by jiadifenolide (150 mg/kg, i.p.), whereas jiadifenolide in this experiment was without significant effect on basal locomotion ($p > 0.05$ compared to vehicle control) (Fig. 4A). Two-way ANOVA of these data confirmed a significant effect of treatment ($F_{3,336} = 65.4$, $p < 0.0001$), time ($F_{11,336} = 31.9$, $p < 0.0001$) and treatment $\times$ time interaction ($F_{33,336} = 2.08$, $p < 0.001$). Post-hoc analysis (Tukey's multiple comparison test) revealed that *d*-amphetamine increased locomotion relative to drug vehicle ($p < 0.0001$) and that jiadifenolide significantly attenuated *d*-amphetamine-induced hyperlocomotion ($p < 0.0001$) with the effects of the drug combination still being greater than vehicle treatment ($p < 0.0001$).

A comparable experiment was conducted with 1 mg/kg picrotoxin (1 mg/kg, s.c.), a dose $\frac{1}{2}$ -log lower than doses that induced convulsions (Fig. 2). In this experiment, picrotoxin significantly attenuated *d*-amphetamine stimulated locomotion of mice (Fig. 4B). Significant effects were observed for treatments ($F_{3,28} = 20.5$, $p < 0.0001$), time ($F_{11,308} = 6.47$, $p < 0.0001$), and treatment $\times$ time interaction ($F_{33,308} = 9.76$, $p < 0.0001$). Post-hoc analysis (Tukey's multiple comparison test) revealed that *d*-amphetamine increased locomotion relative to drug vehicle ($p < 0.0001$) and that picrotoxin significantly attenuated *d*-amphetamine-induced hyperlocomotion ($p < 0.0001$). Although picrotoxin alone did not significantly affect locomotion over time ($p = 0.86$), further time course analysis indicated that at 10–20 min post dosing, picrotoxin significantly decreased locomotion on its own ($p < 0.05$).

3.4. Forced-swim assay.

Mice treated with jiadifenolide (10 or 30 μ M, i.c.v.) showed a tendency ($F_{2,14} = 2.39$, $p = 0.13$ for vehicle and jiadifenolide doses) for greater immobility than that of vehicle control levels (Fig. 5A). In contrast, the antidepressant imipramine (15 mg/kg, i.p.), significantly reduced immobility times (Fig. 5A).

3.5. Body temperature.

Body temperature, measured 30 and 60 min post jiadifenolide dosing (150 mg/kg, i.p.), was not significantly affected (Fig. 5B). Two-way ANOVA confirmed that there was no significant effect of jiadifenolide ($F_{1,16}=0.43$, $p=0.52$), time ($F_{1,16} = 0.43$, $p=0.52$) or time $\times$ drug interaction ($F_{1,16} = 0.02$, $p=0.90$).

3.6. Electrophysiological characterization.

Given the disparate *in vivo* pharmacology of jiadifenolide, picrotoxin, TETS, and bilobalide, we characterized these molecules for their ability to block GABA currents in cultured rat cortical neurons. In this study jiadifenolide and tashironin were shown to be less potent and less efficacious as an antagonist of GABA_A receptors than either TETS, picrotoxin, or bilobalide (Fig. 6).

3.7. Radioligand binding.

To assess potential non-GABA_A receptor interactions of jiadifenolide, radioligand studies were conducted at a single point concentration of 10 μ M. Displacement of standard radioligands of a host of protein targets did not detect significant interaction with these binding sites. The highest displacement was observed with the SERT protein (31.2%) (Table 4).

3.8. Molecular simulation.

Much like the *in vivo* and electrophysiological characterization, the computational simulations of jiadifenolide interactions with a GABA_AR revealed a behavior that is similar, yet less potent, than TETS and picrotoxin. Of the three simulations completed, two resulted in accumulation of jiadifenolide density within the GABA_AR pore near the channel blocking site (Fig. 7A). Molecular docking studies also carried out on this system agreed with the positioning of this potential binding site at the extreme lower end of the pore. This site is in a comparable location to the region identified for both TETS and picrotoxin binding [25]. The majority of the jiadifenolide interactions with the GABA_AR occurred at the -1' ring of the M2 helix (Fig. 7B and C), with minor interactions occurring at the 2' position.

4. Discussion

Despite their potentially valuable therapeutic effects, limited understanding of the biological effects of neurotrophic sesquiterpenes has prevailed due to the complexity of chemical synthesis. We utilized newly devised synthetic methods [7,8] to create quantities of jiadifenolide for *in vivo* characterization, the results of which are described in the present study.

The initial hypothesis about the *in vivo* effects of jiadifenolide was that it would produce convulsions in mice like that of the structurally-overlapping molecules picrotoxin and anisatin. This hypothesis derived from the increased spiking induced by jiadifenolide in rat cortical neuronal culture, an effect shared by *O*-debenzoyltashironin [8]. However, convulsions were not observed with jiadifenolide as they were with the structurally overlapping compounds, picrotoxin and TETS. In order to understand the marked

differential pharmacology of these compounds in vivo, we carried out additional electrophysiological studies to define the potency and efficacy of these molecules as antagonists of GABA_A receptors, a known convulsant mechanism [31,32].

While the recombinant GABA_A receptor currents recorded were completely antagonized by all compounds tested in this study the potency of antagonists differed substantially; these potencies were inversely associated with the convulsive potential of these compounds. Antagonists with known convulsant activity, picrotoxin and TETS, blocked the GABA_A currents in μM range, confirming previously reported values [30,33]. On the other hand, jiadifenolide, an antagonist with no observed convulsive activity, blocked the GABA_A currents only in the 100 μM range consistent with a previous report [8]. Bilobalide with reported weak association to seizure liability [34] blocked GABA_A currents with moderate potency [35]. No seizure data were available in the experimental literature for tashironin [36]. Considering that the potency of tashironin in our study was similar to the potency of jiadifenolide we would expect no convulsive activity at a comparable dose.

The reduced potency and efficacy of jiadifenolide as an antagonist in vivo might explain these differential pharmacological profiles in vivo. Plasma and brain exposure of jiadifenolide after systemic dosing reached levels activating cultured cortical neurons [8] but calculations of the unbound concentrations of jiadifenolide bring these levels below 10 μM . Thus, reduced free drug exposures of jiadifenolide might add to its inability at doses up to 150 mg/kg, i.p. to induce convulsions in mice. However, we also put jiadifenolide directly into the lateral ventricle of mice up to concentrations of 30 μM , also without convulsions or pro-convulsive signs. Although the lower potency/brain exposure of jiadifenolide might be responsible for the lack of convulsive effects observed in the present study, lower concentrations of jiadifenolide (1 μM) are sufficient for induction of the neurotrophic actions of dendrite outgrowth in cortical neurons [37].

Therefore, translation of cellular neurotrophic activity to neurotrophism in vivo might reveal jiadifenolide to have a wider margin of safety than that of other related compounds. For example, picrotoxin was active in attenuating neural and cognitive function in a transgenic mouse model of Down syndrome [4] and Alzheimer's disease [5]. In these studies, 1 mg/kg picrotoxin was used as a subconvulsant dose. Results from the experiments conducted in the present manuscript indicate that the minimal effective dose for inducing convulsions in male, CF-1 mice was between 1 and 3 mg/kg. Therefore, on the basis of dose, the margin between efficacious and toxic doses of picrotoxin is very low.

11-O-Debenzoyltashironin is another member of the neurotrophic sesquiterpenes, trace plant metabolites that enhance neurite outgrowth in cultured neurons [36]. Structural similarity with anisatin, an epileptogenic sesquiterpene [39], suggests a plausible epileptogenic phenotype in vivo. Such possibility is further supported by recent a report of increased spiking induced by 11-O-Debenzoyltashironin in rat cortical neuronal culture [8]. Othawa and colleagues proposed that the 11-O-Debenzoyltashironin causes neurite outgrowth by a weak but persistent hyperexcitation of neuronal cells upon chronic treatment [8]. The weak block of GABA_A receptor currents reported in this manuscript is consistent with such hypothesis. No in vivo seizure studies have reported to date with either 11-O-

Debenzoyltashironin or tashironin presumably due to lack of sufficient quantity of synthetic material. Chemical syntheses by Danishefsky and Theodorakis was however sufficient to replicate the neurite outgrowth phenotype using the PC12 rat brain tumor-derived cell line [40].

Bilobalide was also distinct from that of TETS and picrotoxin in terms of convulsion liability. The effects of bilobalide as an antagonist of GABA_A receptors has been described previously [35] and verified in the present report. Another point of relevance comes from a report by Sasaki et al. [41]. They reported that oral dosing of bilobalide at the 30 mg/kg dose we used here (at least for 4 days), can increase GABA levels in the brain and hence function as an agonist. Finally, the differential in vivo pharmacology might be explained by lower potency as we have described here. Such potency differences might also account for the fact that although frank convulsions up to doses of 30 mg/kg (p.o.) were not produced in mice, bilobalide facilitated clonic convulsions induced the PTZ in mice. A human case report may be relevant here. In two patients with well controlled epilepsy, recurrent seizures developed within 2 weeks of commencing intake of *G. biloba* extract; the seizures abated after discontinuation of the supplement [42].

Although not active as a convulsant at the doses applied in the present study, jiadifenolide was active, albeit with minimal effects, on locomotion of the same strain of mice. Small decreases in locomotion were observed after both i.p. and i.c.v. dosing. In initial observations of mice dosed with jiadifenolide, mice were still exploring the cage at times when vehicle-treated mice were resting in a group. The decreases observed in the locomotor arena vs. the activation relative to control observed in a group might be the result of a drug effect dependent upon the presence of other mice. This possibility, while interesting, was not followed up in the present series of experiments.

In contrast to the small effects on locomotion induced in mice when administered alone, jiadifenolide attenuated the locomotor stimulant effects of *D*-amphetamine in mice, an effect also observed with another isolate from *Illicium* [6]. Blockade of amphetamine-stimulated locomotion has been used to model potential antipsychotic drugs [43] raising the potential for jiadifenolide in this domain. The mechanism by which jiadifenolide produced this effect is not known. Picrotoxin also suppressed *D*-amphetamine-heightened locomotion but only did so while having effects on locomotion per se, a characterized effect of picrotoxin on locomotion [44]. In contrast, jiadifenolide selectively suppressed *D*-amphetamine-stimulated activity. Further studies will be necessary to establish the mechanisms by which jiadifenolide produces this antipsychotic-like drug signature.

Negative allosteric modulators of GABA_A receptors have shown activity in the forced-swim assay, an assay that detects antidepressant drugs [10]. These have included β -CCE [45] and α -5-containing GABA_A receptor ligands like RY-080 [46]. In contrast to these molecules, jiadifenolide produced a small trend in the opposite direction; instead of decreasing immobility times like antidepressants, jiadifenolide produced a trend to increase immobility). The neurotrophic actions of jiadifenolide [1,2] suggested that it would be active in the forced swim assay that detects the activity of other neurotrophic / antidepressant molecules [3]. In addition, picrotoxin has been shown to be active in this assay [47].

However, subchronic dosing of jiadifenolide might be essential to observe this antidepressant-like effect—an experiment that remains to be conducted. Body temperature was decreased by other molecules derived from *Illicium* (derived from the flower, Star anise) [6]. Body temperature was unchanged with jiadifenolide in the present experiment.

Thus, jiadifenolide might represent a unique compound in the domain of the neurotrophic compounds. However, at present the mechanisms responsible for the relatively benign in vivo pharmacology profile of jiadifenolide are uncertain. Although it exhibits modest potency and efficacy at GABA_A receptors, and the unexceptional brain exposures observed in the present study might be held to account for its tolerability, other possibilities need to be tested. Jiadifenolide has been screened against a battery of neuronal receptors and not found to robustly displace any radioligands in this panel (present study). Of course, given the multiple binding sites at each receptor, this data must be approached cautiously. For example, the radioligand for GABA_AR in this panel is [³H]-muscimol, whose binding would not be strongly affected by a weak channel binder.

However, both jiadifenolide and *O*-debenzoyletashironin were assayed for displacement of the channel binder [³H]-TBOB, and only *O*-debenzoyletashironin exhibited competitive binding ($K_i = 61 \mu\text{M}$). Given the demonstrated functional activity of both compounds at GABA_AR, jiadifenolide might bind a site distinct from TBOB, which shares a similar binding site to *O*-debenzoyletashironin, picrotoxin, bilobalide and TETS. However *O*-debenzoyletashironin, jiadifenolide, or picrotoxin do not demonstrate disparate selectivity for GABA_A alpha subunits 1, 2, 3, or 5 [8] suggesting that alpha subtype selectivity does not determine functional differences in pharmacology. However, multiple other possibilities could be responsible. For example, some yet unidentified unique protein target for jiadifenolide could guide its novel pharmacological properties. Nonetheless, evaluation of 44 protein targets in the present study did not uncover any one with remarkable potency.

A naïve target identification campaign has not been carried out for jiadifenolide or *O*-debenzoyletashironin but would be of great value if affinity labels can be identified that do not affect the neurotrophic phenotype. Additionally, the simulation data reported in the present paper suggests a potential site in the pore/M2 helix region that is at an overlapping, yet lower position than those observed for other ‘cage convulsant’ compounds such as TETS, EBOB, and picrotoxin [30] (Fig. 7C). One could hypothesize that a position nearer to the entrance of the pore channel may allow for easier displacement of jiadifenolide from its blocking location leading to lower potency. Furthermore, the presence of many similar, overlapping regions of jiadifenolide density at the pore entrance suggests a ‘looser’, lower affinity binding interaction to pore which allows the compound to move around and occupy different positions. The orientation of jiadifenolide indicates that the carbonyl oxygens are in a similar same alignment to the GluCl crystal structure of picrotoxin [48]. However, the long axis of the molecule is horizontal, making deeper pore entrance more difficult. A more detailed structural characterization campaign may help to resolve these questions. Mutagenesis studies could help to identify specific binding domains.

In the meantime, it is worth noting an analogy with the NMDA receptor ion pore. Memantine, is an uncompetitive channel blocker with low affinity for the receptor (~1 μM).

It has been suggested that the low affinity of memantine for the receptor (characterized as a low trapping blocker) gives this compound therapeutic advantage over other NMDA receptor antagonists [49]. Higher affinity ligands such as MK-801 or phencyclidine (nM) induce marked psychotomimetic effects accompanied by motor impairment consisting of marked incoordination and psychomotor stimulation in rodents and humans [50,51]. In contrast, memantine presents with a markedly reduced side-effect profile [52]. This safety profile enabled memantine (Namenda) to become one of the only drugs available for symptomatic treatment of dementias where it is sometimes given in conjunction with acetylcholinesterase inhibitors [53]. Like the binding of jiadifenolide described in the current manuscript, memantine binds to a unique site on the NMDA receptor complex that creates low binding affinity and ease of dissociation [49].

Similar to jiadifenolide, bilobalide is an antagonist at GABA_A receptors, yet exhibits beneficial cognitive effects at low to moderate dose. The effects of both bilobalide and jiadifenolide might derive from their antagonism of tonic versus phasic activation of GABA_A receptors [54] and this possibility has not yet been interrogated. The recent identification of the ion channel 'desensitization' gate [55], its proximity to the picrotoxinin binding site, and the potential for channel binders to differently affect current kinetics, conspire to make the possibilities endless. It is plausible that jiadifenolide affects tonic conductance but its binding is kinetically out-of-synch with phasic conductance.

Given the potential therapeutic utility of such compounds and the use of bilobalide as a natural product supplement, a molecule with neurotrophic actions that has reduced toxicity liabilities is advantageous. Systematic replication of the neurotrophic actions of jiadifenolide and extension of this work to in vivo endpoints of therapeutic interest (e.g., cognitive improvement, antidepressant activity, neuroprotective actions, etc.) is needed. In the meantime, advances in synthetic routes to these complex natural products [7,8] satisfy the material demands for such scientific inquiry.

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Non-Standard Abbreviations

AMPA	α -amino-3-hydroxy-5-methyl- 4-isoxazolepropionic acid
AP-4	4-Aminopyridine
CTZ	cyclothiazide
PAM	positive allosteric modulator

PI	protective index
PTZ	pentylene-tetrazole
TETS	tetramethylenedisulfotetramine

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Figure 1.
Structures of the molecules studied.

Figure 2.

Heat map showing effects of the drugs on behaviors of CF-1 mice. Each mouse (ordinate) was assigned the highest heat rating that individual exhibited during each time period (abscissa). * alert/active/ambulating/digging vs. huddled together resting or sleeping as were vehicle control mice at the same time period.

Figure 3.

Effects of jiadifenolide given by the intraperitoneal route (**A**) or by intracerebroventricular route (**B**) on locomotion of male, CF-1 mice. Each point or bar represents the mean $\pm$ S.E.M. of 8 mice. * $p < 0.05$ compared to vehicle by Dunnett's test.

Figure 4.

A. Effects of jiadifenolide (150 mg/kg, i.p.) alone and in combination with d-amphetamine (2 mg/kg, i.p.) on locomotor activity of CF-1 mice. **B.** Effects of picrotoxin (1mg/kg, s.c.) alone and in combination with d-amphetamine (2 mg/kg, i.p.) on locomotor activity of CF-1 mice. Each point represents the mean $\pm$ S.E.M. of the same 8 mice over time.

Figure 5.

A. Effects of jiadifenolide on immobility time in the forced swim test in male, NIH Swiss mice. Each bar represents the mean $\pm$ S.E.M. of 8 mice. Imi=imipramine (15 mg/kg, i.p.). * $p < 0.05$ by post-hoc Dunnett's test. **B.** Effects of jiadifenolide (150 mg/kg, i.p.) on rectal body temperature of CF-1 mice. Each bar represents the mean $\pm$ S.E.M. of 5 mice.

Figure 6.

Antagonism of recombinant GABAA-currents recorded on IW_Barracuda automated patch instrument. (A) Effects of neurotrophic (Jiadifenolide, Tashironin, Bilobalide) and convulsive (Picrotoxin, TETS) terpenes on the peak amplitude of 300μM GABA-evoked currents in cultured HEK293 expressing recombinant GABAA receptors ($\alpha 1\beta 3\gamma 2$). The data are plotted as mean + S.E.M and the curves are fitted with 4-parameter Hill equation. (B) The table summarizes the mean IC₅₀ values, Hill slopes and the number of replicates for each compound tested.

Figure 7.

A. The simulation system of the GABAA Receptor (cyan) embedded in a phospholipid bilayer (lipid headgroups shown as red balls with lipid fatty acids as white tails). Water, counter ions, and the nearest two GABAA Receptor subunits have been removed for clarity. The regions of highest average jiadifenolide density from two separate simulations are represented as green and yellow masses. **B.** A close-up view of the pore region shows the jiadifenolide density relative to the 6', 2', and -1' rings of residues of the M2 helix. **C.** The representative binding mode for jiadifenolide bound to the GABA_A receptor (top) shows partial overlap with an experimentally-derived binding site observed for picrotoxin bound to the structurally homologous GluCl1 (bottom). Three of the M2 helices are represented as grey ribbons. All hydrogens have been removed for comparison to the crystal structure. Carbon atoms of the ligands are shown in green, and oxygen atoms in red.

Table 1.

Effects of jiadifenolide alone and in combination with pentelenetetrazole.

Treatment ^I	Clonus	Tonus	Lethality
Jiadifenolide + vehicle	0/10	0/10	0/10
Vehicle + PTZ 45 mg/kg	0/5	0/5	0/5
Jiadifenolide + PTZ 45 mg/kg	1/5	0/5	0/5
Vehicle + PTZ 70 mg/kg	4/5 *	0/5	0/5
Jiadifenolide + PTZ 70 mg/kg	4/5 *	0/5	0/5

^I Jiadifenolide (150 mg/kg) was dosed i.p., 30 min prior to second injection. PTZ= pentelenetetrazole. PTZ was dosed s.c.

* p<0.05 compared to Jiadifenolide + vehicle by Fisher's Exact Probability test.

Table 2.

Effects of bilobalide alone and in combination with pentelenetetrazole

Treatment ^I	Clonus	Tonus	Lethality
Bilobalide+ vehicle	0/8	0/8	0/8
Vehicle + PTZ 60 mg/kg	3/13	0/13	0/13
Bilobalide + PTZ 60 mg/kg	11/13 [*]	0/13	0/13

^I Bilobalide was dosed at 30 mg/kg, p.o., 60 min prior to second injection. PTZ= pentelenetetrazole. PTZ was dosed s.c.

^{*} p<0.05 compared to Biloboline + vehicle by Fisher's Exact Probability test.

Table 3

Plasma and brain concentrations (total and calculated unbound [u] concentrations) along with the brain to plasma ratios or Kpu for intraperitoneally-dosed jiadifenolide. Data are means and (S.D.) of 3 mice per dose group

Dose (mg/kg, i.p.)	[Plasma] (μM)	[Brain] (μM)	Brain to Plasma ratio	Calculated [plasma]u (μM)	Calculated [brain] u (μM)	Kpu, u
30	0.671 (0.188)	3.200 (0.390)	5.47	0.329	1.44	4.3
150	12.1 (2.77) **	25.8 (12.8) *	1.96	5.93	11.61	1.95

* p < 0.05.

** p < 0.01 by unpaired-*t*-test relative to 30 mg/kg value.

Table4Displacement of radioligand binding by jiadifenolide (10 μ M).

Receptor	Percent displacement	Receptor	Percent displacement
5-HT1a	6.4	D3	-8.3
5-HT1b	19.7	D4	1.6
5-HT1d	3.2	D5	28.8
5-HT2a	13.2	DAT	3.4
5-HT2b	10.4	DOR	-5.6
5-HT2c	15.2	GABAA	5.1
5-HT3	-11.2	H1	9.4
5-HT5a	13.5	H2	-8.3
5-HT6	14.4	H3	-9.7
5-HT7	-18.8	H4	-5.3
Alpha1a	3.1	KOR	5.2
Alpha1b	25.7	M1	-15.4
Alpha1d	16.1	M2	-10.5
Alpha2a	9.6	M3	0.9
Alpha2b	-15.7	M4	-11.3
Alpha2c	-12.6	M5	-18.8
Beta1	-10.6	MOR	-7.3
Beta2	0.1	NET	25.6
Beta3	-8.1	PBR	-10.1
BZP rat brain site	-17.4	SERT	31.2
D1	17.2	Sigma1	-11.5
D2	-26.4	Sigma2	9.6

Data represent mean % inhibition (N = 4 determinations) for compound tested at receptor subtypes.

Significant inhibition is considered where displacement is > 50%. In cases where negative inhibition is observed, this represents stimulation of binding. Data were obtained from the National Institute of Mental Health's Psychoactive Drug Screening Program. Methods are available at <https://pdspdb.unc.edu/pdspWeb>.