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ORIGINAL RESEARCH

Ferulic acid dimer as a non-opioid therapeutic for acute pain

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Purpose: Search for alternate pain medications has gained more importance in the past few years due to adverse effects associated with currently prescribed drugs including nervous system dysfunction with opioids, gastrointestinal discomfort with nonsteroidal anti-inflammatory drugs, and cardiovascular anomalies with cyclooxygenase-2 (COX-2) inhibitors. Phytomedicine has been explored for the treatment of pain, as these have been used for generations in regional communities and tend to lack major side effects in general. One such phytomedicine, incarvillateine (INCA), derived from the Chinese herb *Incarvillea sinensis* has its primary antinociceptive action through the adenosine receptor, a novel pain target. We hypothesized that derivatives of cinnamic acid dimers, which are structurally similar to INCA, would show potent antinociceptive action and that their effect would be mediated through adenosine receptor action.

Materials and methods: Dimers of cinnamic acid (INCA analogs) were synthesized using cavitand-mediated photodimerization (CMP) method, which utilizes a macromolecule (γ-cyclodextrin) to control excited state reactivity of photoactive compounds. Acute pain response was assessed by using formalin-induced licking behavior in hind paw of mice, and neurologic function was monitored through locomotor activity, mechanical hyperalgesia, and thermal sensitivity upon administration of test compound. For mechanistic studies, binding to adenosine receptor was determined by using computer modeling.

Results: Ferulic acid dimer (FAD), which has the same chemical functionalities on the aromatic ring as INCA, showed significant suppression of formalin-induced acute pain. Antinociceptive effect was observed primarily in the inflammatory phase, and no apparent behavioral changes related to the nervous system were noticeable. Inhibition of opioid receptor did not reverse antinociceptive response, and modeling data suggest adenosine 3 receptor binding.

Conclusion: FAD (INCA analog) shows potent nonopioid antinociceptive action mediated predominantly through A_3AR – adenosine 3 receptor action. Further characterization and selection of such INCA analogs will help us generate a new class of antinociceptives with precise chemical modifications by using CMP methodology.

Keywords: adenosine, incarvillateine, cinnamic acid, formalin, antinociceptive

Introduction

Chronic pain is a debilitating condition that affects more people than cancer, diabetes, and heart disease combined. It commonly occurs post-surgery, accompanies several other lingering diseases, and is a leading cause of long-term disability.[1] Treatment for pain has been an ongoing area of research despite a plethora of current medications including opioid antagonists, cyclooxygenase-2 (COX-2) inhibitors, nonsteroidal

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anti-inflammatory drugs (NSAIDs), and to some extent tricyclic antidepressants.[2] Recently, there is a major emphasis on research for alternate pain medications due to neurological dysfunction with opioids, gastrointestinal discomfort with NSAIDs, and cardiovascular anomalies with COX-2 inhibitors. Several studies emphasize the importance of phytomedicine for the treatment of pain, as these have been used for generations in regional communities tested and proven over time and tend to lack major side effects.[3,4]

Incarvillateine (INCA) is an active compound from the Chinese herb, *Incarvillea sinensis*, which has traditionally been used to treat rheumatism and relieve pain.[5–7] Research on antinociceptive actions of INCA in chronic and acute pain models indicate that diphenyl-dicarboxylic acid-substituted cyclobutane core (active core) is essential for its medicinal property.[7–9] This finding spurred research to synthesize INCA analogs, which are structural (stereo-/regioisomeric, Figure 1) variants of the active core with some additional chemical functionalities such as alkyl, hydroxy, alkoxy, and halogen groups and study their antinociceptive properties; phytocompounds containing this active core are referred to as truxillic and truxinic acid depending on their structures (Figure 1).

Synthesis of INCA analogs by using classical organic chemistry transformations is inefficient,[6,10,11] as it involves multiple synthetic steps, which generate significant waste and reduce overall yield. On the contrary, an excited state reaction known as photodimerization of alkenes (cinnamic acid [CA] in this case) could afford these compounds in good yields (Figure 2), but the reaction suffers from very poor conversion as the excited state molecules are typically short-lived (lifetime: 10^{-6} s). INCA analogs are known as dimers, as they are obtained from the combination of two CA monomers.

Synthetic INCA analogs reported in literature have been limited to just two stereoisomers: *anti* head-to-tail (*anti* H-T) and *syn* head-to-head (*syn* H-H) of some CAs. A grand total of <30 compounds (including derivatives) have been investigated thus far.[5,7,8,12] However, for any one given CA, the four stereogenic carbon centers and relative arrangement of the groups on the cyclobutane core could lead to as many as 11 isomeric structures.[13] Thus, there is significant drug discovery potential if INCA analogs with specific steroisomeric structures could be produced.

Our group specializes in the synthesis of these dimers by including the two CAs within larger molecules with hollow spaces (cavitands) to increase the chance of encounter in excited state. This method known as cavitand-mediated photodimerization (CMP, Figure 3) is effective in producing stereospecific dimers in significant yields.[14–16] CMP can thus produce a wide variety of cinnamic acid dimers (CADs) and is more effective than solid-state photodimerization approach used by other groups, which is applicable only to specific reactants.[12] As will be discussed below, the *syn* H-H ferulic acid dimer (FAD) was synthesized in preparative scale by

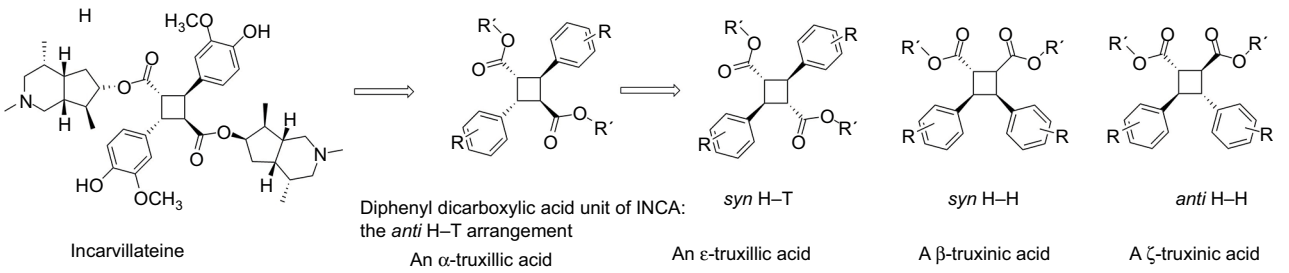

Figure 1 Structure of incarvillateine (INCA), the diphenyl dicarboxylic acid core in INCA, its isomeric structural variants, and their corresponding common names (truxillic and truxinic acids).

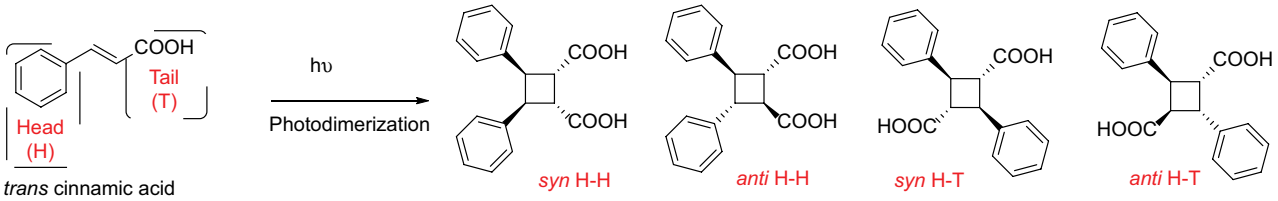

Figure 2 Photodimerization of *trans* cinnamic acid and the four possible isomeric dimers.

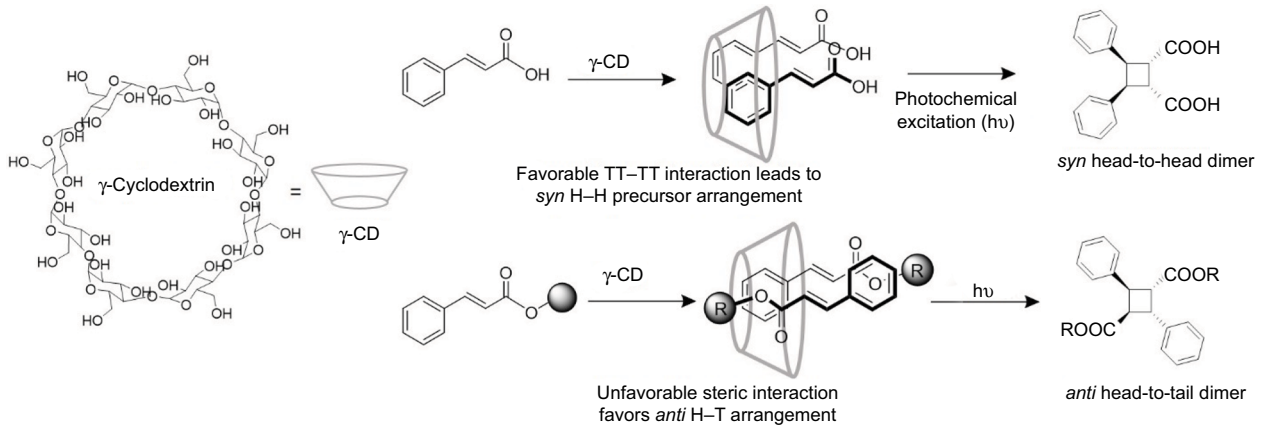

Figure 3 Cavitand-mediated photodimerization (CMP) method for synthesis of cinnamic acid dimers.

using CMP. Other methods involving irradiation of crystals of ferulic acid obtained from several solvents did not result in any dimerization: they were all photo-inert in solid state.

In search of a new class of antinociceptives, adenosine-mediated analgesia has become an attractive option as it has shown minimal central nervous system (CNS)-related effects.

Adenosine is increased in sites of tissue injury due to breakdown of adenosine triphosphate, and thus receptors involved with adenosine action ($A_{2a}AR$, A_3AR) get upregulated in these regions.[17,18] Adenosine receptors (A_1AR, $A_{2a}AR$, $A_{2b}AR$, A_3AR) are widely distributed in spinal cord and brain areas involved in the sensation of pain. A_1AR was considered as the best target for the development of analgesics; however, cardiovascular effects with full agonists has raised a concern and thus increased the research toward considering partial agonists or allosteric modulators.[19,20] Agonists to $A_{2a}AR$ and $A_{2b}AR$ significantly suppress autoimmune neuroinflammation as well as sepsis, though exhibiting some peripheral pronociceptive and pro-inflammatory effects.[17,20,21] Several preclinical studies have been performed with A_3AR agonists, with actions on glial cells, which have been quite effective in suppressing neuropathic pain.[18,20,22,23] INCA has been shown to act primarily through the adenosine receptors (A_1AR and A_2R) to reduce chronic inflammatory and neuropathic pain,[8] but involves opioid receptors (μ and κ) in addition to adenosine receptors in an acute formalin-induced pain model.[5]

The reason INCA has not been explored widely for use as an antinociceptive is because of its rare occurrence and complex synthesis procedures. Our aim in this study was to screen CA dimers with different substitutions for their antinociceptive properties and identify analogs with potent action at the adenosine receptor. We report here an innovative one-step CMP method to synthesize CA dimers (INCA analogs), which were further characterized for their pharmacological properties in alleviating pain. From the compounds that we have analyzed thus far, *syn* H-H FAD has been shown to be the most effective antinociceptive agent and appears to work through adenosine pathway; FAD has the same aromatic structural units as INCA.

Materials and methods

Animals

Adult CD1 male and female mice (20–25 g) were purchased from Charles River Laboratories (Kingston, NY, USA) and allowed to acclimate to the environment before using them for experiments. Animals were kept in a facility with controlled temperature and humidity regulation; food and water were provided ad libitum. All procedures were performed based on the approval of the respective protocols from Institutional Animal Care and Use Committee at the University of Nebraska-Kearney (#060916) and University of Cincinnati (#041416). Institutional guidelines for the proper and humane use of animals for research were followed. As formalin is toxic to tissues, mice were euthanized immediately after the procedure by using carbon dioxide inhalation, followed by cervical dislocation. Experiments were performed when the animals were between 6 and 12 weeks old.

Chemicals

All chemicals (ferulic acid, formalin, polyethylene glycol, dimethyl sulfoxide [DMSO], and naloxone) were purchased from Millipore Sigma (St. Louis, MO, USA). CA dimers were synthesized by using CMP method (described below). Before use, investigational drugs were dissolved in 10% volume of DMSO, and 40% volume of polyethylene glycol (PEG3000) was added to stabilize the complex. The drug

solution was further diluted in 0.9% saline to make a working stock (15–20 mg/mL), which was then used to inject the animals per their body weight. Saline control was prepared by similar method excluding the drug (10% DMSO, 40% PEG, and rest 0.9% saline).

Synthesis of photodimers

CMP method was used to produce FADs. About 100 mg of β-truxinic acid was added to half equivalent of γ-cyclodextrin (γ-CD, mol wt 1297 g/mol) in a 20 mL Pyrex vial containing 15 mL of water. The reaction mixture was stirred on a hotplate for 2 h, at ~70ºC. After turning off the heat, the mixture was further allowed to stir at room temperature for 6 h. As complexation occurs, the 2:1 (ferulic acid:γ-CD) complex precipitates out resulting in a homogeneous slurry. The precipitate was then filtered off, washed with 10 mL of cold water and 10 mL of cold ethanol to remove any uncomplexed ferulic acid or γ-CD and allowed to air-dry for 12 h. The resulting dry lumps of precipitate (complex) were ground to obtain powder of even consistency. This powder was then placed between glass plates and irradiated in a photochemical irradiation chamber fitted with medium-pressure mercury vapor lamp for about 96 h. The glass plates were periodically flipped to ensure complete exposure of the reactants to UV light. After irradiation, the complex was dissociated through biphasic extraction by using water–ethyl acetate mixture wherein the complex was added to 100 mL of water and 100 mL of ethyl acetate, stirred for 2 h, and the two layers were separated. The ethyl acetate layer was collected, dried using magnesium sulfate, and solvent removed in a rotary evaporator. The product was purified by recrystallization in ethyl acetate–dichloromethane (90:10) mixture and analyzed using ^{1}H nuclear magnetic resonance (NMR) spectroscopy (400 MHz, D6-acetone). The presence of two quazi doublet signals between 3 and 5 ppm confirmed the presence of dimers. The *syn* H-H dimer was obtained in yields >70%, and the product was purified by recrystallization from ethyl acetate. Purity of compounds thus obtained was >95% based on NMR integrations. The product structure for ferulic acid *syn* H-H dimer (not reported in literature yet) was confirmed by using X-ray crystallography; single crystal of diffraction quality of FAD was grown from a mixture of ethyl acetate in dichloromethane. The structures of other dimers have been previously established by the authors and ascertained using ^{1}H NMR spectra.[14–16]

Acute pain testing model

Investigational drug was administered intraperitoneally (ip) in the lower left abdomen of the mouse by using a 27.5-G needle (BD Biosciences, San Jose, CA, USA). Acute inflammatory pain was induced by intraplantar injection of 20 µL of 5% formalin in the left hind paw, 10 min after the administration of the drug, by using a 30-G needle (BD Biosciences). Time spent licking the left hind paw was observed and recorded for both the early phase (0–5 min) and the late phase (5–50 min). Indomethacin (30 mg/kg, ip) was used as positive control for inflammatory pain reduction pathway. Experimenters were blinded to the identity of the test compound, while performing the study to reduce any bias. To prevent formalin-associated toxicity to the tissues, animals were used only once and euthanized immediately after the experiment.

Locomotor studies

A Rota-rod system (IITC Life Science Inc., Woodland Hills, CA, USA) was used to assess the locomotor function in mice. Mice were trained on the Rota-rod system before performing the test. Drug or vehicle was injected 1 h prior to testing the mice. Each mouse was tested for three trials separated by 10-min intervals.[24,25] During the tests, the speed of rotation was accelerated from 5 to 35 rpm in 3 min. The falling latency was recorded and averaged.

Mechanical hyperalgesia (Von Frey) test

Mechanical hyperalgesia was assessed through the measurement of paw withdrawal threshold by using the "Up-and-Down" paradigm, as previously described.[26,27] Briefly, the mice were first acclimatized (1 h) in individual clear plexiglass boxes on an elevated wire mesh platform to facilitate access to the plantar surface of the hind paws. Von Frey filaments of increasing stiffness (0.02–4 g) were applied to the hind paw plantar surface of the animals with a pressure high enough to bend the filament. The 50% mechanical paw withdraw threshold (threshold 50%), expressed in grams was evaluated before (Basal) and 1 h after treatment with FAD or vehicle.

Heat sensitivity (Hargreaves) test

For testing heat sensitivity, mice were placed in plastic boxes, and the hind paw withdrawal latency to Hargreaves radiate heat apparatus (IITC Life Science) was measured. A cutoff of 20 s was set to prevent potential tissue damage.[28] The latency, expressed in seconds, was evaluated before (Basal) and 1 h after treatment with FAD or vehicle.

Docking studies for receptor binding

FAD structure was docked into adenosine A_3AR receptor (PDB code 1OEA) by using AutoDock 4.2.6 software and the PyRx[29] interface. The grid box was set with 25, 35, and 20 points in the *x*, *y*, and *z* directions.

X-ray crystallography

A Bruker Venture instrument with Cu-IμS source was used for single-crystal X-ray diffraction. Integrated intensity information was obtained by reduction, merging, and scaling of data frames with APEX3 and corrected for absorption by using ADABS (BRUKER AXS Inc., Madison, WI, USA). Systematic reflection conditions and statistical tests of the data suggested the space group *P*-1. A solution was obtained readily by using XT/XS in APEX3. Hydrogen atoms were placed in idealized positions and were set riding on the respective parent atoms. All non-hydrogen atoms were refined with anisotropic thermal parameters. Elongated thermal ellipsoids on O6-C17 group indicated disorder which was modeled successfully between two positions with an occupancy ratio of 0.51:0.49. Appropriate restraints were added to keep the bond distances, angles, and thermal ellipsoids of the disordered group meaningful. Absence of additional symmetry or void were confirmed by using PLATON (ADDSYM).[42] The structure was refined (weighted least squares refinement on F^2) to convergence. Olex2 was used for the final data presentation and structure plots.[43]

Statistical analysis

Animal experiments were performed with five animals per treatment group. The three Rs for animal research – reduction, refinement, and replacement – were considered while performing the experiments. Animal numbers were restricted to five per group unless there were occasional outliers, when the number was increased to six per group. All values obtained are expressed as mean ± SEM. Results shown were analyzed with GraphPad Prism version 7.03 for Windows (GraphPad Software Inc., La Jolla, CA, USA). Statistical comparison was performed by using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test (for multiple group, one factor) or two-way ANOVA followed by Tukey's multiple comparison test (for multiple groups, multiple factors). Differences were considered to be statistically significant at $p<0.05$.

Results

Synthesis and characterization of CAD

The CADs used in this study were synthesized by using CMP method (Figure 2). Four isomeric dimers (Figure 3) could result from direct photodimerization depending on the relative orientation of the alkenes while reacting in solution phase, which restricts the yield of one specific dimer.

The relative orientation of CAs within the cavity of γ-CD is a result of intermolecular interactions. When CAs are included within γ-CD cavity, a head-to-head (H-H) orientation is favored due to stabilizing/attractive hydrogen bonding (between the carboxylic acids of two CAs) and π-π (between phenyl rings of two CAs) interactions. Irradiation of this complex yielded the *syn* H-H dimer (Figure 4). On the contrary, when carboxylic acid functionalities were converted to esters (especially isopropyl), destabilizing steric interaction between bulky alkyl groups and lack of attractive hydrogen bonding interaction flipped the relative orientation to a head-to-tail (H-T) orientation (Figure 4). Thus, irradiation of alkyl cinnamates yielded the *anti* H-T dimers. All the dimers synthesized showed structural similarity to INCA (Figure 4). Structure of *syn* H-H FAD was confirmed by using X-ray crystallography (Figure 5).

Effect of FAD in reducing acute pain

Antinociceptive properties of ferulic acid monomer (FAM) and FAD were tested by using formalin-induced acute pain model in mice. As shown in Figure 6, early-phase licking response (0–5 min) did not show marked change with vehicle control or any of the drugs used. On the contrary, late-phase licking response (5–50 min) was markedly elevated in vehicle-treated mice, which was reduced to a similar extent by using FAD (*syn* H-H) or indomethacin (a known anti-inflammatory drug). The *anti* H-T FAD or isopropyl CAD was not as effective as the *syn* H-H isoform. All the drugs were used at 30 mg/kg concentration and injected 10 min prior to introducing formalin in hind paw.

Dose–response effects of FAD on antinociceptive response in acute pain model

FAM has shown antinociceptive effects in some models of neuropathic pain primarily induced through nerve injury or administration of chemotherapeutic agents, although we did not observe such response in the male mice at the dose used.[30,31] On the contrary, FAD showed a maximum suppression of late-phase pain response at the 30 mg/kg dose (Figure 7), which was subsequently used for studying gender differences and receptor responses.

Gender differences in acute pain response and effects of FAM and FAD

Both male and female mice showed similar intensity to formalin-induced pain in the early and late phases (Figure 8B). Interestingly, FAM was able to suppress late-phase pain response in female mice (30 mg/kg) although it did not show any such effect in male mice. FAD treatment showed similar pain reduction in male (Figure 8A) and female (Figure 8B) mice.

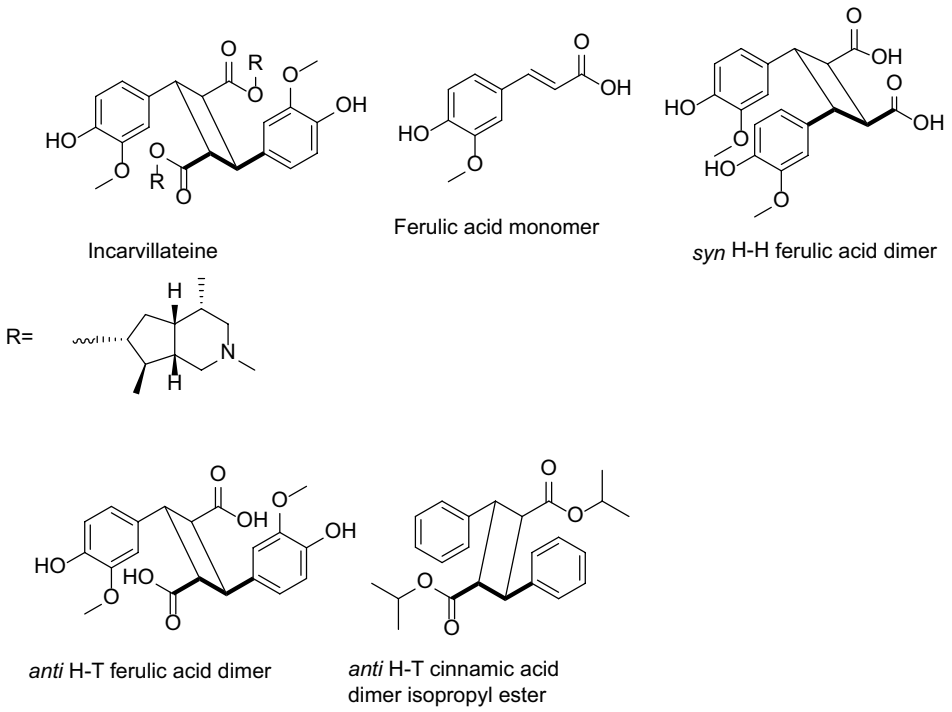

Figure 4 Structure of incarvillateine – and other analogs used in this study.

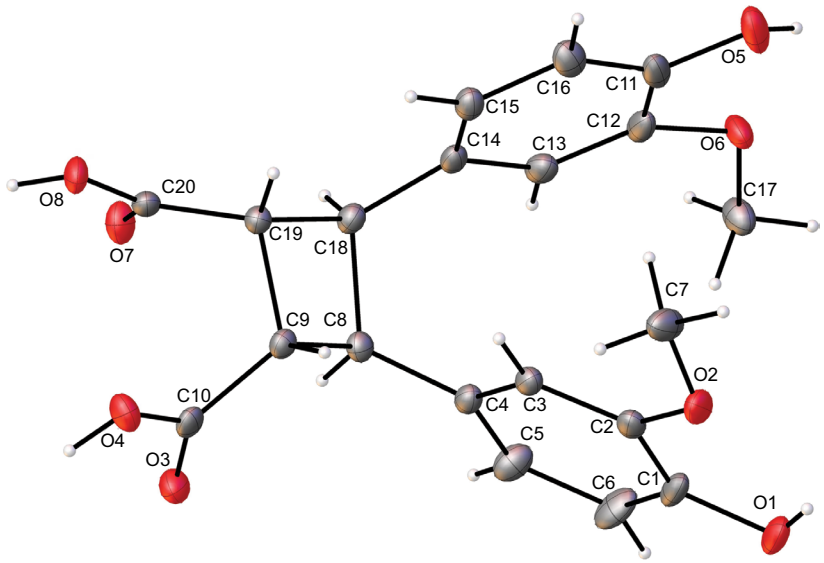

Figure 5 X-ray crystallography structure of ferulic acid dimer (*syn* H-H). Carbons (gray), hydrogens (white), and oxygens (red).

Effect of FAD administration on thermal sensitivity, mechanical hyperalgesia, and locomotor functions

Both male and female mice showed normal nociceptive response upon FAD administration as monitored by using mechanical hyperalgesia (Figure 9A and B) and thermal sensitivity (Figure 9C and D). In addition, Rota-rod function test suggested lack of FAD-induced locomotor impairment in both male and female mice (Figure 9E and F). These results indicate that FAD maintains normal neurologic functions in mice.

Mechanistic action of FAD on opioid receptor system

Opioid receptor antagonist, naloxone (2 mg/kg, ip)[32] was administered prior to FAD to understand the involvement of opioid pathway. Naloxone did not reverse the antinociceptive

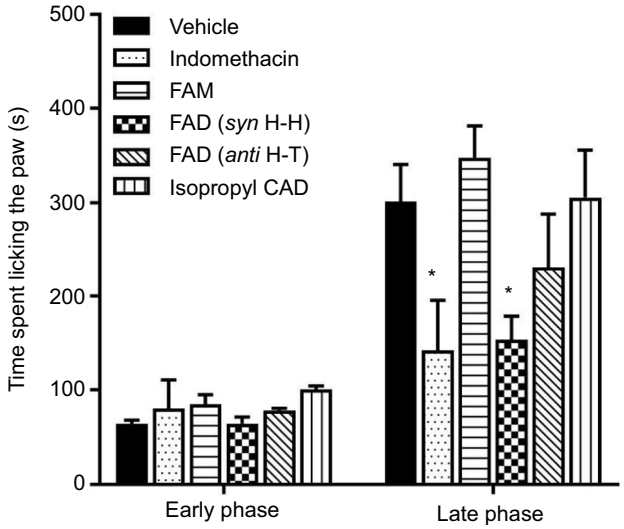

Figure 6 Effect of indomethacin, ferulic acid monomer (FAM), ferulic acid dimer (FAD), *syn* H-H, *anti* H-T, and isopropyl CAD (all drugs at 30 mg/kg, ip) on formalin-induced acute pain in left hind paw of male mice (n=5 animals per group, *p<0.05 compared to vehicle control for late-phase response).

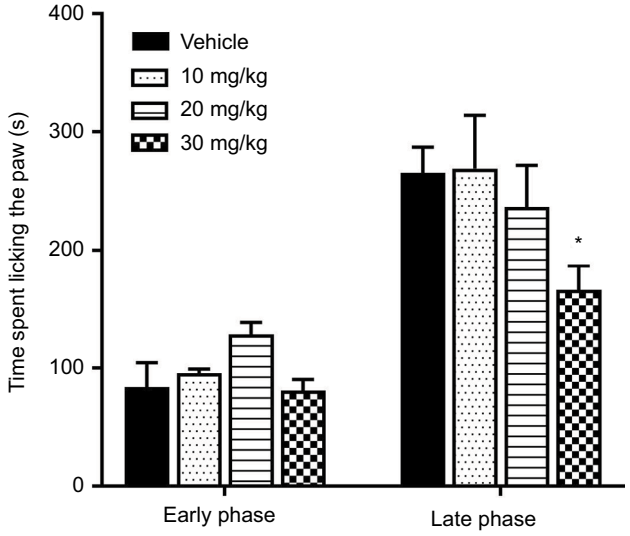

Figure 7 Dose–response with ferulic acid dimer (FAD; 10, 20, and 30 mg/kg, ip) on formalin-induced acute pain on left hind paw in male mice (n=5 animals per group, *p<0.05 compared to vehicle control for late-phase response).

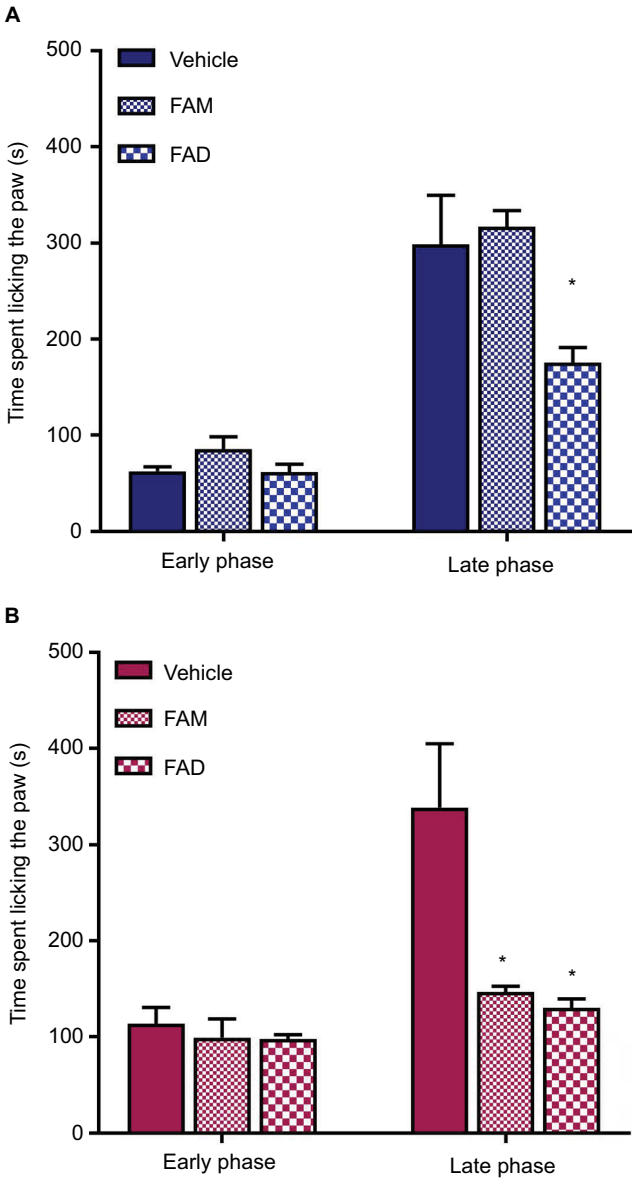

Figure 8 Effect of ferulic acid monomer (FAM, 30 mg/kg, ip) and ferulic acid dimer (FAD, 30 mg/kg, ip) on formalin-induced acute pain on left hind paw in male (**A**) and female (**B**) mice (n=5 animals per group, *p<0.05 compared to vehicle control for late-phase response).

action of FAD; the response in both early and late phases were very similar to FAD (Figure 10).

Molecular modeling of FAD with adenosine 3 receptor (A_3AR)

INCA has been shown to bind both opioid and adenosine receptors, although adenosine receptor action appears to be the prominent one. As A_3AR has been the most effective adenosine receptor for pain reduction,[33] we tested the molecular docking analysis of FAD with this receptor. A homology model of A_3AR (PDB code 1OEA) was used to explore the binding site and environment of receptor bound to FAD (Figure 11). Docking analysis was performed with AutoDock software through the PyRx interface. Diaryl cyclobutyl dicarboxylic acid core resides in the upper region of the TM (transmembrane helix) bundle showed several interactions. Phenolic hydroxy group is involved in tight hydrogen bond network with the side chain of Asn250 and Gln167. Hydrophobic interaction established by Val61, Val65, and Ser165 anchors the dicarboxylic acid in the binding pocket. An additional hydrophobic interaction stabilizes the aromatic ring of dimer compound with Leu271. The phenyl ring and

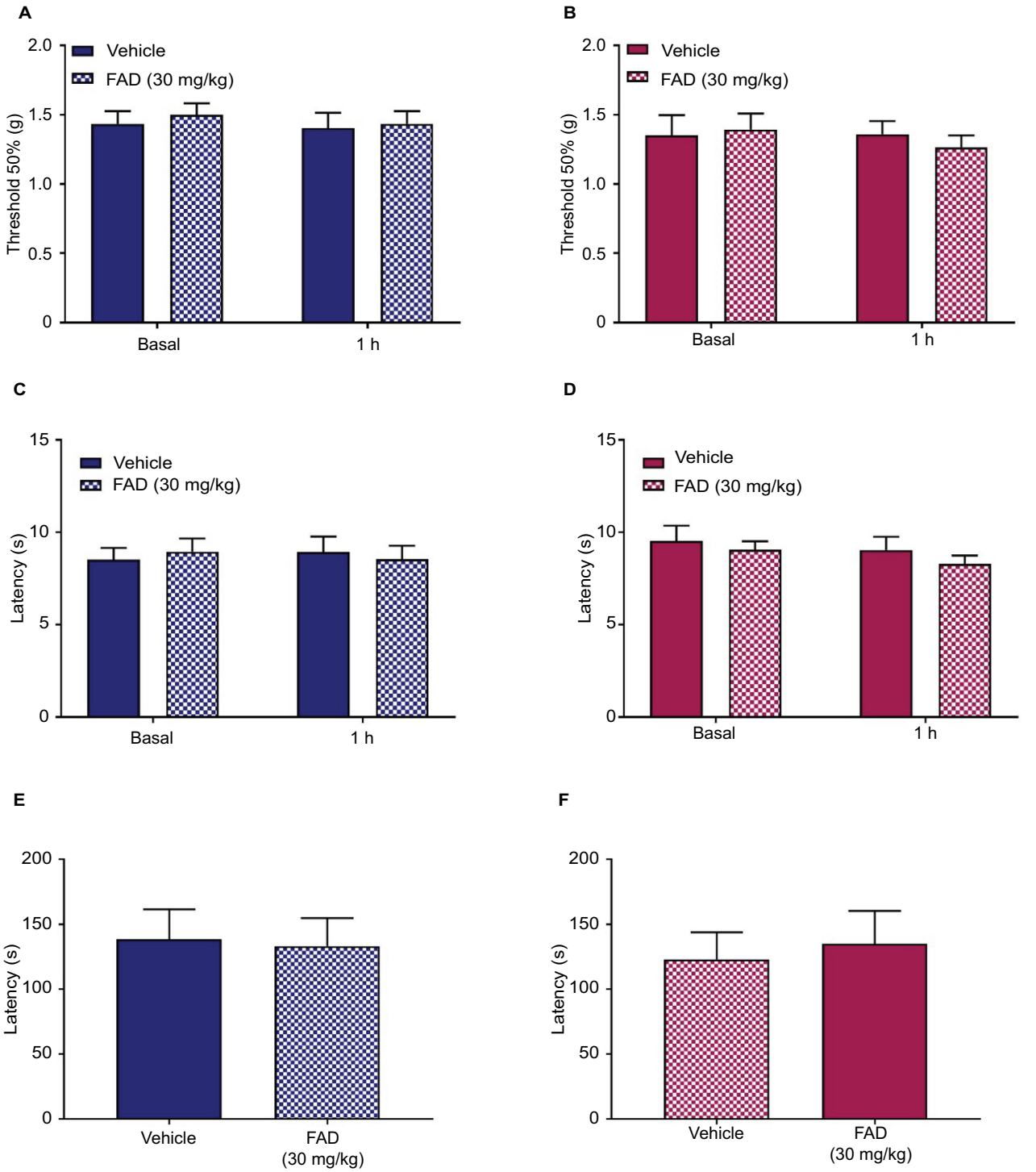

Figure 9 Nervous system function tests with ferulic acid dimer (FAD) administration. Effect of FAD on mechanical hyperalgesia (Von Frey test) in (**A**) male and (**B**) female mice. The 50% mechanical paw withdraw threshold (threshold 50%), expressed in grams was evaluated before (Basal) and 1 h after treatment with FAD or vehicle control. Effect of FAD on thermal sensitivity (Hargreaves test) in (**C**) male and (**D**) female mice. The latency, expressed in seconds, was evaluated before (Basal) and 1 h after treatment with FAD or vehicle control. Effect of FAD on locomotor function (Rota-rod test) in (**E**) male and (**F**) female mice. The latency, expressed in seconds, was evaluated before (Basal) and 1 h after treatment with FAD or vehicle control. The falling latency was recorded and averaged (n=5 animals per group for all tests).

carboxylic acid group are located in proximity to His272 and Ser271. The OMe and OH substituent at the phenyl ring is oriented toward the Thr94 and involved in H-bonding. Strong hydrophobic interaction of the cyclobutyl ring of dimer is observed with hydrophobic residues Ile268 and Leu246. The conformation adopted by FAD-A_3AR complex revealed several interactions in the present model, and this is in good agreement with the data published previously.[34,35] Homology binding to other adenosine receptors was not as evident as with A_3AR.

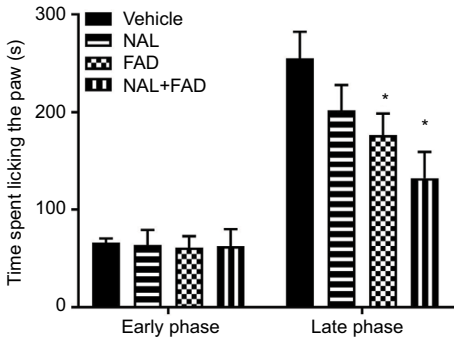

Figure 10 Effect of ferulic acid dimer (FAD) on opioid system. General opioid receptor blocker, naloxone (NAL, 2 mg/kg, ip) was administered 20 min before injecting FAD, followed by formalin procedure (n=5 animals per group, *p<0.05 vs vehicle late-phase control).

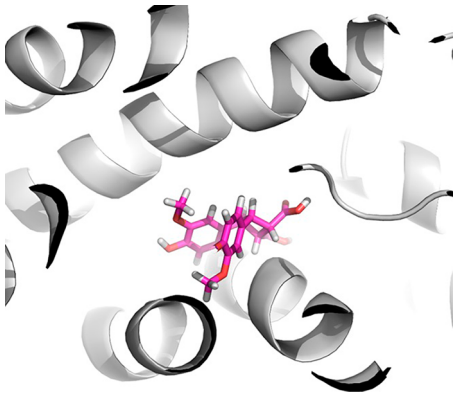

Figure 11 Ferulic acid dimer (FAD) (pink, tube model) with homology model of adenosine A_3AR receptor (PDB code 1OEA).

Discussion

Research on alternative pain medications is on a steady rise due to concerns with advancing opioid epidemic and lack of an effective pain-suppressing agent. Phytocompounds, used in traditional medicine for pain reduction, have gained great attention due to their advantage of lack of neurologic side effects including issues related to tolerance and dependency. In the current study, CADs were synthesized, which structurally mimic INCA, an active component from the Chinese herb, *I. sinensis*.

CAs predominantly undergo unimolecular photoisomerization upon irradiation to yield *cis* CA in solution. Total synthesis of CADs through classical organic synthetic routes is tedious and involves at least seven synthetic steps with moderate to low yields in each step, which also result in impractically low yields if the preparative scale compounds are needed for animal testing. Photodimerization occurs when a photoexcited alkene encounters another alkene in the ground state, which is a low-probability event. Our laboratory takes advantage of macrocyclic molecules with cavities (cavitands), such as γ-CD, to include two CAs within its cavity. Due to proximity and pre-orientation factors during the photochemical reaction, high yields (~70%) of desired photodimers were achieved. This method has been used in producing stereospecific dimers from more than 15 different CAs so far.[15,16,36] Our previous studies have demonstrated that CAs that fail to dimerize in solid state afford high yields of dimers when subjected to CMP method. Ferulic acid undergoes near quantitative conversion with CMP and affords high yields; on the contrary, crystals of ferulic acid obtained from ethyl acetate, ethanol, methanol, isopropanol, acetone, acetonitrile/methanol (50:50), toluene/acetone (50:50), and ethanol/water (80:20), are photo inert and did not undergo any photodimerization at all. This method circumvents the complexity in synthesizing or extracting INCA[5–8,37] and tedious classical synthesis procedures for CADs. X-ray crystallography of *syn* H-H FAD that was generated displayed the characteristic central cyclobutane ring, with its other substituents, as observed in INCA.

FAD (*syn* H-H) significantly suppressed formalin-induced acute inflammatory pain in mice with 30 mg/kg concentration being the most effective, and this concentration was subsequently used for further experiments. Similar concentrations have previously been reported with INCA for acute and chronic pain studies.[5,8,38] The *anti* H-T conformation of FAD showed some alleviation of pain in the late phase but was not significant compared to vehicle control. Early phase pain response (0–5 min) with formalin is indicative of neurogenic pain, whereas late phase (5–50 min) refers to inflammatory pain. Morphine, an opioid drug, suppresses both neurogenic and inflammatory pain response, although indomethacin (Cox-2 inhibitor drug) acts on the inflammatory phase response only.[5] FAD (*syn* H-H) showed antinociceptive action similar to indomethacin in the late phase with little to no effect in the early phase, which indicates a primary non-opioid action. Research has shown that INCA binds to both opioid and adenosine receptor, although its primary action is through the adenosine pathway.[8]

Ferulic acid is a phenolic phytochemical found ubiquitously in seeds and leaves of plants.[39] It has shown several biological properties including antioxidant, anticancer, analgesic, antiviral, and vasodilatory.[39] Previous reports refer to antinociceptive action of ferulic acid (monomer, FAM) through the opioid pathway, as it has shown positive results in neurogenic pain models.[30,39] In the current study, FAM did not suppress acute pain in male mice although it was as potent as FAD in alleviating late-phase pain in female mice. Several reports have suggested that there is a clear sex difference

in pain perception and pain inhibition in both human and mouse models.[40,41] Theories explaining these sex differences range from variations in neurological connections, genetics, psychological, and neurochemicals in males versus females. Thus, future studies will further investigate structure–activity relationships of CADs and their respective monomers for possible gender differences in pain relief, which could help in the process of customizing patient care.

INCA has been reported to mediate its antinociceptive effects by binding to both opioid and adenosine receptors, although its primary action is believed to be through the adenosine receptor, specifically adenosine 1 and 2a receptors (A_1AR, $A_{2b}AR$).[8] As indicated earlier, FAM has been shown to suppress neurogenic pain via opioid receptor action.[30,39] With the experiments performed so far, we believe that FAD does not work through the opioid pathway as it suppresses only inflammatory response in formalin model and did not show any apparent neurogenic locomotor impairment, and opioid antagonist did not reverse its antinociceptive effect. In addition, mechanical hyperalgesia and thermal sensitivity tests indicate that normal nociception was retained in mice after FAD treatment, which suggests that neurologic functions are maintained. Due to lack of crystal structures for all receptors, computational docking analysis of FAD was performed with A_3AR, which indicates strong binding interactions with this receptor. Our aim in the future is to synthesize CADs with primary adenosine receptor action, as they have shown minimal neurogenic side effects.[18,21,33] Mechanistic studies to understand biological action at the receptor and cellular signaling pathways for pain suppression with FAD will provide further insight into its range of action.

Conclusion

We have synthesized and identified a CAD analog, which has potent antinociceptive properties in formalin-induced acute pain and binds strongly to one of the adenosine receptors. The prospect of its action through adenosine pathway (a non-opioid mechanism) is highly advantageous in pursuing research on a novel class of potent antinociceptives which can be transitioned for preclinical drug trials.

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Author contributions

All authors contributed to the experimental design, data collection and analysis, and drafting and revision of the manuscript. They agree to be responsible for all aspect of the work.

Disclosure

The authors report no conflicts of interest in this work.

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