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Original Article

Addictive evaluation of cholic acid-verticinone ester, a potential cough therapeutic agent with agonist action of opioid receptor

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Aim: The purpose of this work was to search for potential drugs with potent antitussive and expectorant activities as well as a low toxicity, but without addictive properties. Cholic acid-verticinone ester (CA-Ver) was synthesized based on the clearly elucidated antitussive and expectorant activities of verticinone in bulbs of *Fritillaria* and different bile acids in Snake Bile. In our previous study, CA-Ver showed a much more potent activity than codeine phosphate. This study was carried out to investigate the central antitussive mechanism and the addictive evaluation of CA-Ver.
Methods: Testing on a capsaicin-induced cough model of mice pretreated with naloxone, a non-selective opioid receptor antagonist, was performed for the observation of CA-Ver's central antitussive mechanism. We then took naloxone-induced withdrawal tests of mice for the judgment of CA-Ver's addiction. Lastly, we determined the opioid dependence of CA-Ver in the guinea pig ileum.
Results: The test on the capsaicin-induced cough model showed that naloxone could block the antitussive effect of CA-Ver, suggesting the antitussive mechanism of CA-Ver was related to the central opioid receptors. The naloxone-urged withdrawal tests of the mice showed that CA-Ver was not addictive, and the test of the opioid dependence in the guinea pig ileum showed that CA-Ver had no withdrawal response.
Conclusion: These findings suggested that CA-Ver deserved attention for its potent antitussive effects related to the central opioid receptors, but without addiction, and had a good development perspective.

Introduction

Hubeibeimu, listed in Pharmacopoeia of the People's Republic of China (Chp)2000 and Chp2005, is the dried bulb of *Fritillaria hupehensis* Hsiao *et* KC Hsia. Within the realm of Chinese medicine, Hubeibeimu has been claimed to resolve phlegm, stop cough, and clear away heat, and dispel accumulation; it is widely used in Traditional Chinese Medicine. Recent pharmacological studies demonstrated that the total and single alkaloids (*eg*, verticinone) of this medicine had antitussive activity[1]. Verticinone is the main alkaloid of *F hupehensis* Hsiao *et* KC Hsia, comprising about 0.3% of the crude herb. Verticine, the other main alkaloid of *F hupehensis*

Hsiao *et* KC Hsia, is the alcohol derivative of verticinone. Verticine is easily oxidized to verticinone. Together, the two make up over 80% of the total alkaloids. Shedan is a valuable Chinese crude drug. Systematic chemical and pharmacological research studies have confirmed that its main components are taurocholic acid and various kinds of free cholic acids[2–4].

Enlightened with the "combination principle" in drug discovery, we synthesized cholic acid-verticinone ester (CA-Ver), a novel derivative of verticinone and cholic acid. The pharmacodynamic studies on the antitussive and expectorant activities of CA-Ver were then screened with different animal models. CA-Ver showed a much more potent antitussive activity than codeine phosphate. A further acute toxicity study showed that the LD_{50} value of CA-Ver exceeded 3.5 g·kg^{-1} by intraperitoneal injection in mice. CA-Ver showed a potential future in the development of a new antitussive drug.

In a previous study[5], we assayed the influences of CA-Ver on five kinds of monoamine neurotransmitters in mouse brain, using reverse-phase HPLC method with a fluorescent light detector[6, 7] and with codeine and dextromethorphan as positive drugs[8]. The results indicated that the antitussive mechanism of dextromethorphan concerned the monoamine neurotransmitters 5-HT. Further, dextromethorphan could increase the amount of 5-HT in the animal's brain to produce antitussive effects, while the antitussive effects of CA-Ver had no relationship with serotonergic mechanisms, just as codeine. This study was carried out to investigate the central antitussive mechanism of CA-Ver. Codeine, a centrally acting, narcotic antitussive drug, is one of the most commonly used potent antitussive agents; however, addiction to codeine limits the clinical usefulness of this agent[9]. In order to determine whether CA-Ver is addictive like codeine, naloxone-urged withdrawal tests of mice were performed for the observation of addiction to CA-Ver. We then undertook a method for the quantitative determination of opioid dependence in the guinea pig ileum *in vitro* to confirm an absence of addiction to CA-Ver[10, 11].

Materials and methods

Preparation of CA-Ver CA-Ver (Figure 1) was synthesized from cholic acid and verticinone *via* esterification, using dicyclohexylcarbodiimide (DCC) as the acid-activating agent and leading to the linkage of the carboxylic acid group on the C24 position of cholic acid, with the hydroxyl group on the C3 position of verticinone. The synthesis of CA-Ver is illustrated in Figure 2 (five steps, 33% overall yield). The reaction of cholic acid with absolute MeOH followed by esterification resulted in the formation of methyl 3α,7α,12α-trihydroxy-5β-cholan-24-oate. Then, the reaction of **1** with tert-butyl dimethyl chlorosilane (TBDMSiCl)[12] *via* ether bond afforded the intermediate **2**. Hydrolysis of the methyl ester groups of **2** with 5% sodium hydroxide aqueous solution produced **3**. Then treatment of **3** with verticinone in CH_2Cl_2, followed by esterification, gave the compound 5α,14α-cevanin-6-*O*-20β-hydroxy-3β-yl-3α-tert-butyldimethylsilyloxy-7α,12α-dihydroxy-5β-cholan-24-oate, **4**. In this reaction, 4-dimethylaminopyridine (DMAP) was used as a catalyst and *N,N'*-dicyclohexylcarbodiimide (DCC) as a dehydration agent[13]. Finally, the deprotection reaction of **4** in 5% HF[14] aqueous solution gave the desired 5α,14α-cevanin-6-*O*-20β-hydroxy-3β-yl-3α,7α,12α-trihydroxy-5β-cholan-24-oate, **5**. The chemoselective esterification reaction of **3** with verticinone in CH_2Cl_2 provided **4** in 58.9% yield.

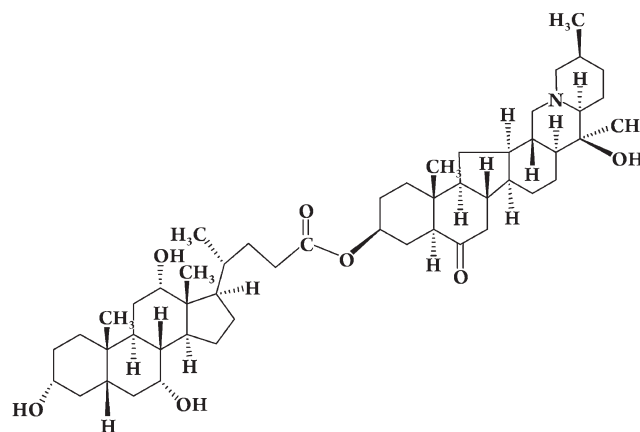**Chemicals** The CA-Ver was synthesized by us (the

Figure 1. Structure of CA-Ver. (5α,14α-cevanin-6-*O*-20β-hydroxy-3β-yl-3α,7α,12α-trihydroxy-5β-cholan-24-oate)

purity of CA-Ver was determined by HPLC to be ≥98.0%). The codeine phosphate and morphine hydrochloride were purchased from the Qinghai Pharmaceutical Factory (Qinghai, China). The naloxone hydrochloride injection was purchased from the Chengdu Beite Pharmaceutical Factory (Chengdu, China). Acetylcholine chloride (99%) was obtained from Sigma-Aldrich. Verticinone was isolated from *F hupehensis* Hsiao *et* KC Hsia, which is commercially available from the Hubei Institute of Chinese Materia Medica (Wuhan, China) and was identified by Prof De-tai PENG (Lichuan Institute of Chinese Materia Medica, Lichuan, Hubei province, China). A voucher specimen (F030823) was deposited at the Faculty of Pharmaceutical Science, Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). The capsaicin was purchased from the Fluka reagent company (EC № 2069698, Purity ≥97.0% by HPLC).

Animals Kunming mice of both sexes (Experimental Animal Center, Tongji Medical College, Huazhong University of Science and Technology, China), weighing about 30−40 g, were used for the test on capsaicin-induced cough model, and mice weighing about 13−17 g were used for the naloxone-urged withdrawal test. Male Dunkin-Hartley guinea pigs (300−350 g, Experimental Animal Center, Tongji Medical College, Huazhong University of Science and Technology, China) were killed by CO_2 inhalation. All mice and guinea pigs had free access to food and water, and were maintained in a designated animal room, at 24±1 °C and with a 12-h light-dark cycle. This study was carried out in accordance with the Declaration of Helsinki and/or the "Regulation for the Administration of Affairs Concerning Experimental Animals" (State Council of China, 1988). Minimum numbers of animals were used to result in mean-
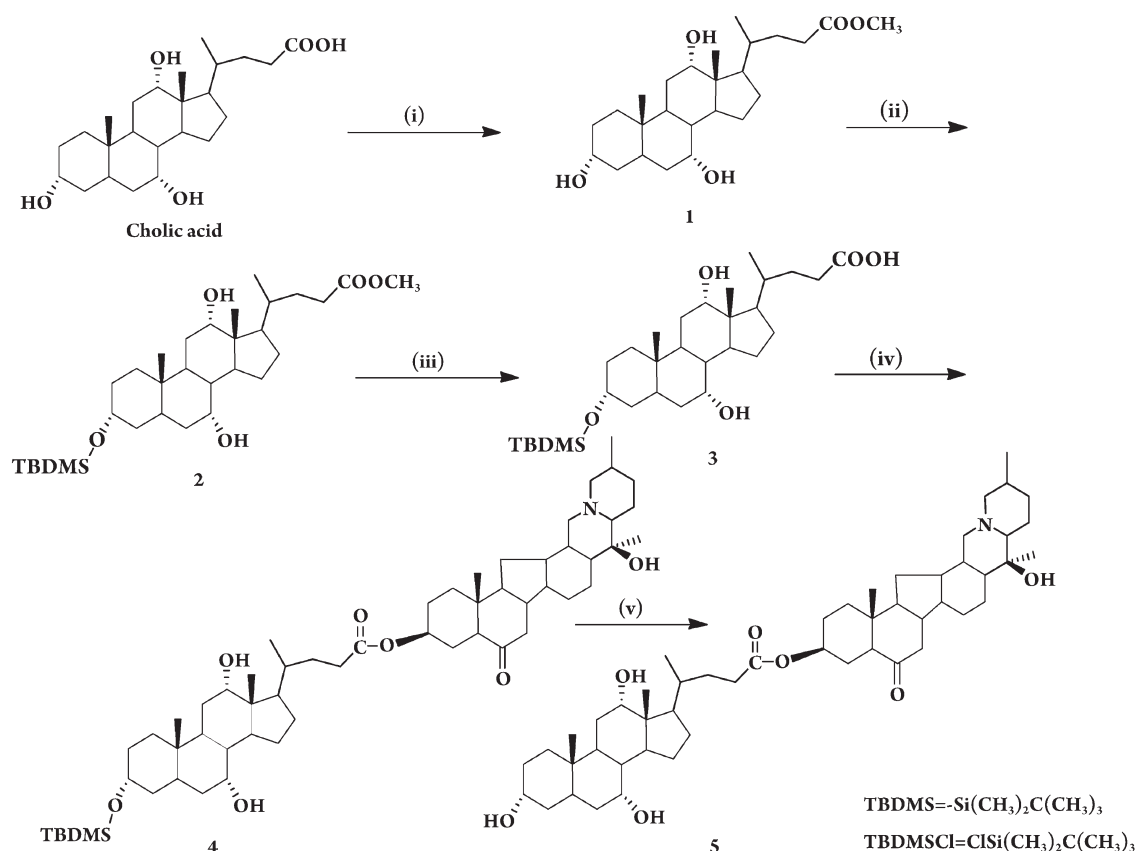
Figure 2. Reagents and conditions. (i) MeOH/concentrated sulfuric acid, room temperature (rt) 1 day; (ii) TBDMSCl/imidazole/THF, low temperature, 48 h; (iii) 1: 10% NaOH/THF, rt, 12 h; 2: H₂O/H⁺, rt, 30 min; (iv) verticinone/DCC/DMAP/CH₂Cl₂, rt, 48 h. (v) 5% HF(aq)/THF, rt, 24 h.

ingful interpretation of data, and animal discomfort was kept to a minimum.

Antitussive assay The cough reflex was induced as previously described by Kamei[15, 16]. Briefly, the unanesthetized animals were placed individually in a transparent tailor-made bottle (dimensions: 10 cm×5 cm×5 cm) and exposed to a nebulized solution of capsaicin (100 μmol/L) for 5 min using a body plethysmograph. The same nebulizer was used throughout the experiment. Capsaicin was dissolved in 10% ethanol and 10% Tween 80 saline solution at a concentration of 0.15 mg/mL, and the mixture was diluted with saline. The coughs produced during a 5 min exposure period were counted. The animals were selected by the number of coughs 72 h before the test. Animals with more than 30 or fewer than 8 coughs in a 5 min period were not used for the studies. After 72 h of recovery, the selected mice were randomly divided into several groups, with at least ten animals in each group, for different treatments. The CA-Ver and codeine phosphate were suspended in 0.5% carboxyl

methylcellulose solution and were administered orally. The mice were exposed to capsaicin for 5 min beginning 30 min before the administration of antitussive agents to determine the frequency of the control coughs. The animals were again exposed to capsaicin aerosol for 60 min after the administration of the antitussive agents. The number of coughs produced after the drug administration (C_t) was compared with the number of control coughs (C_c). The antitussive effect was expressed as the percentage of inhibition of the number of control coughs $[(C_c-C_t)/C_c]\times100\%$.

The naloxone-urged withdrawal tests of mice We used naloxone-urged withdrawal tests of mice for the judgment of addiction to the CA-Ver[17, 18]. In this study, we assayed the addiction to CA-Ver using codeine phosphate and morphine hydrochloride as the positive drugs. All drugs were mixed with quantitative forage powders, and then the mixtures were pressed into newly made animal feeds. The production of animal feed mixed with codeine is described as an example of the process. First, 1600 mg codeine phosphate was ground

to a powder and mixed with 800 g of forage powder by the method of the isometric increasing principle. Then, we added 800 mL of distilled water to the mixture. Finally, we passed the farina through a 5 mL syringe to produce a strip, which was dried, first at room temperature for 24 h, then at 50 °C for 24 h. Other animal feeds mixed with different drugs were made with the same procedure as described for codeine phosphate, but with different dosages. Mice were divided into five groups of ten animals. Group 1 animals (Control) were treated with the animal feeds without drug for 15 days. Group 2 was treated with codeine phosphate as the positive drug. Group 3 was treated with morphine hydrochloride and Group 4 was treated with verticinone. Group 5 was treated with CA-Ver. Each mouse's body weight was measured at 9:00 am daily. Each group was fed with forage plus drugs for 15 days. Each mouse's body weight was recorded at 12:00 am on the 16th day. Immediately thereafter, naloxone (4 mg/kg) was injected (ip) and the mouse was put on a 30 cm diameter frustum at a height of 35 cm for observation. During the 30 min observation period, the mouse was watched continuously by a trained observer and the frequency of jumps was recorded. At 30 min, the mouse was once again weighed, fed with forage without drugs, and then weighed every 30 min, until the mouse's body weight recovered.

The test of opioid dependence in the guinea pig ileum *in vitro* Guinea pigs were deprived of food for 24 h and killed by CO_2 inhalation. Pieces of ileum were immediately removed from a point within 10 cm from the ileo-caecal junction. These pieces were dissected and placed in Krebs-Henseleit solution (mmol/L): NaCl, 112.08; KCl, 5.90; $CaCl_2$, 1.97; $MgCl_2$, 1.18; NaH_2PO_4, 1.22; $NaHCO_3$, 25.00; and glucose, 11.49. The ileum was placed under 1 g of tension in a 5 mL organ bath containing the nutrient solution, at 37 °C, with continuous bubbling of 95% O_2 and 5% CO_2. After 60 min equilibration in Krebs-Henseleit solution, the contraction tension of the ileum was recorded by a multi-channel physiological signal-gathering processing system (RM-6280C, Chengdu Instrument Factory, Chengdu, China). At the start of each experiment, a maximum response to acetylcholine (3 μmol/L) in each tissue was obtained to check its stability. Dependence, as indicated by a strong contraction of the ileum when challenged with naloxone, was produced by incubating the ileum from a native guinea pig with opioid at 37 °C for 4 h[10, 11]. The response of the ileum to naloxone was time-dependent and directly related to the codeine phosphate concentration in the incubation fluid (10–1 000 nmol/L) and to the challenge dose of naloxone (10–1 000 nmol/L)[19]. The guinea pig ileum was

incubated with drugs at 37 °C for 4 h. The drug concentration in the incubation fluid was 3 μmol/L, and the challenge dose of naloxone was 3 μmol/L. The withdrawal responses were expressed as the ratio to the acetylcholine (3 μmol/L) responses. The mean amplitude of the stimulated contraction by naloxone was about 50% of the maximal response to acetylcholine (3 μmol/L). The dependence of CA-Ver was determined by comparing it with the effects of codeine phosphate in the ileum.

Statistical analysis In all studies, the values were expressed as means±SEM, of *n* observations. The results were analyzed by one-way analysis of variance followed by a *Bonferroni post-hoc* test for multiple comparisons. *P*<0.05 was considered significant.

Results

Antitussive effect of CA-Ver on capsaicin-induced coughs CA-Ver, at doses of 1.5, 3, and 6 mg/kg, *po*, dose-dependently inhibited the number of capsaicin-induced coughs when the antitussive effect was examined 60 min after administration (Figure 3).

Effects of naloxone on the antitussive effect of CA-Ver The antitussive effect of CA-Ver (6 mg/kg, *po*) was abolished by pretreatment with naloxone (4 mg/kg, ip). As shown in Figure 4, the pretreatment with naloxone, a non-selective opioid receptor antagonist, significantly (*P*<0.05) reduced the antitussive effect of codeine phosphate, a centrally acting, narcotic antitussive drug (with saline, 62.18%±8.14%, *n*=10; with naloxone, 35.03%±10.93%, *n*=10). The antitussive effect of CA-Ver was also significantly (*P*<0.05) antagonized by naloxone (with saline, 60.15%±10.54%, *n*=10; with naloxone, 35.60%±9.68%, *n*=10) (Figure 4).

Addictive evaluation of CA-Ver in mice The number of jumps and the percentage of weight loss in the mice in the CA-Ver group were not significantly different from the negative control group, but were both less than the two positive control groups (*P*<0.01). The percentage of jumping animals in the CA-Ver group was similar to the negative control group, but lower than the positive control groups. The drug dosages administered to the mice were based upon the median lethal doses (LD_{50}) of the different drugs. The dose of codeine phosphate and morphine was 400 mg·kg^{-1}·d^{-1} and 200 mg·kg^{-1}·d^{-1}, respectively. However, the dose of verticinone was only 6 mg·kg^{-1}·d^{-1} and CA-Ver was 200 mg·kg^{-1}·d^{-1}. As shown in Table 1, the naloxone urged withdrawal tests of mice showed that CA-Ver was not addictive.

Dependence evaluation of CA-Ver in the guinea pig ileum *in vitro* Segments of the ileum taken from the same
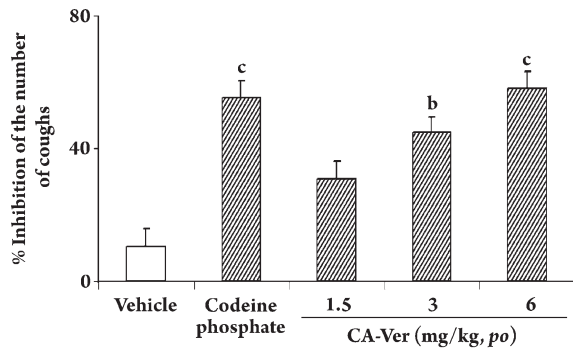
Figure 3. Dose-response data for the antitussive effect induced by CA-Ver in mice. The antitussive effects were assessed 60 min after the administration of each drug. Each point or column represents the mean±SEM for ten mice in each group. [b]$P<0.05$, [c]$P<0.01$ vs the value for the respective vehicle-treated group (open column). The dose of codeine phosphate was 30 mg/kg. CA-Ver and codeine phosphate were suspended in a 0.5% carboxyl methylcellulose solution. Vehicle: 0.5% carboxyl methylcellulose solution.
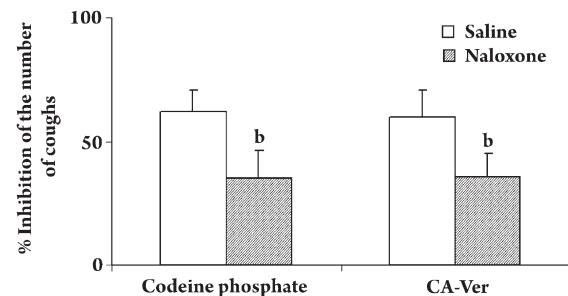

Figure 4. Effects of naloxone on the antitussive effect of codeine phosphate and CA-Ver in mice. Naloxone (4 mg/kg) was injected (ip) 45 min after the administration of codeine phosphate and CA-Ver. The antitussive effects of codeine phosphate (30 mg/kg) and CA-Ver (6 mg/kg) were assessed 60 min after oral administration. Each column represents the mean±SEM for ten mice in each group. [b]$P<0.05$ vs the respective saline-treated group (open column).

native guinea pig, were each incubated in Krebs solution containing a different drug (codeine phosphate 3 μmol/L, verticinone 3 μmol/L, cholic acid 3 μmol/L, and CA-Ver 3 μmol/L) at 37 °C for 4 h. The withdrawal contracture was elicited by a challenge with naloxone (3 μmol/L). As shown in Figure 5, in the ileum incubated in Krebs solution with codeine phosphate for 4 h, the addition of naloxone induced a withdrawal contracture. As shown in Table 2, the codeine phosphate group showed a significant difference from negative control group ($P<0.01$). But the CA-Ver group showed no significant difference from the negative control group. Taken together with the naloxone-urged withdrawal tests of mice, our results indicated that CA-Ver induced no addiction.

Table 1. Number of naloxone-urged jumps and the weight decrease ratio of mice. $n=10$. Mean±SEM. [c]$P<0.01$ vs vehicle; [e]$P<0.05$ vs codeine phosphate group; [i]$P<0.01$ vs morphine hydrochloride group; Vehicle: purified water.

Group	Dose/ mg·kg^{-1}·d^{-1}	Number of jumps	Rate of jumping animals/%	Weight decrease ratio/%
Vehicle	–	0.50±0.71	40	3.38±2.04
Codeine phosphate	400	6.20±6.06[c]	80	5.93±0.57[c]
Morphine hydrochloride	200	8.40±6.79[c]	100	6.55±1.54[c]
Verticinone	6	1.30±3.09[ei]	40	3.08±3.49[ei]
CA-Ver	200	0.10±0.32[ei]	10	4.58±1.30[ei]

Table 2. Naloxone (3 μmol/L)-precipitated withdrawal responses of the guinea pig ileum incubated with different drugs at 37 °C for 4 h. Withdrawal responses %=withdrawl contracture/acetylcholine contracture×100%. $n=10$ guinea pigs. Mean±SEM. [c]$P<0.01$ vs vehicle; [f]$P<0.01$ vs codeine phosphate group. Vehicle: purified water. All other drugs were dissolved in purified water.

Group	Dose/μmol·L^{-1}	Withdrawal responses/%
Vehicle	–	4.7±4.6
Codeine phosphate	3	50.4±17.1[c]
Verticinone	3	3.2±4.1[f]
Cholic acid	3	5.6±3.5[f]
CA-Ver	3	1.9±2.9[f]

Discussion

Cough remains among the most common complaints for which patients seek medical attention. Presently available therapies to treat cough are limited due to a lack of effective medications. Moreover, most existing antitussive drugs can bring inevitable or intolerable side effects; codeine or dextromethorphan, for example, have limited efficacy in chronic cough at the recommended doses, and unacceptable side effects at higher doses[20–22]. The most pressing and unmet current need is for safe and more effective cough suppression[23]. To search for potential drugs with potent antitussive and expectorant activities as well as low toxicity but no addiction, CA-Ver was synthesized based on the clearly elucidated antitussive, expectorant, and antiasthmatic activities of verticinone in bulbs of Fritillaria and different bile acids in Snake Bile.

Capsaicin, the active ingredient of red pepper, is a commonly used cough stimulant in the study of cough reactivity and for the evaluation of antitussive agents[24, 25]. It has been shown that capsaicin induces cough mainly by
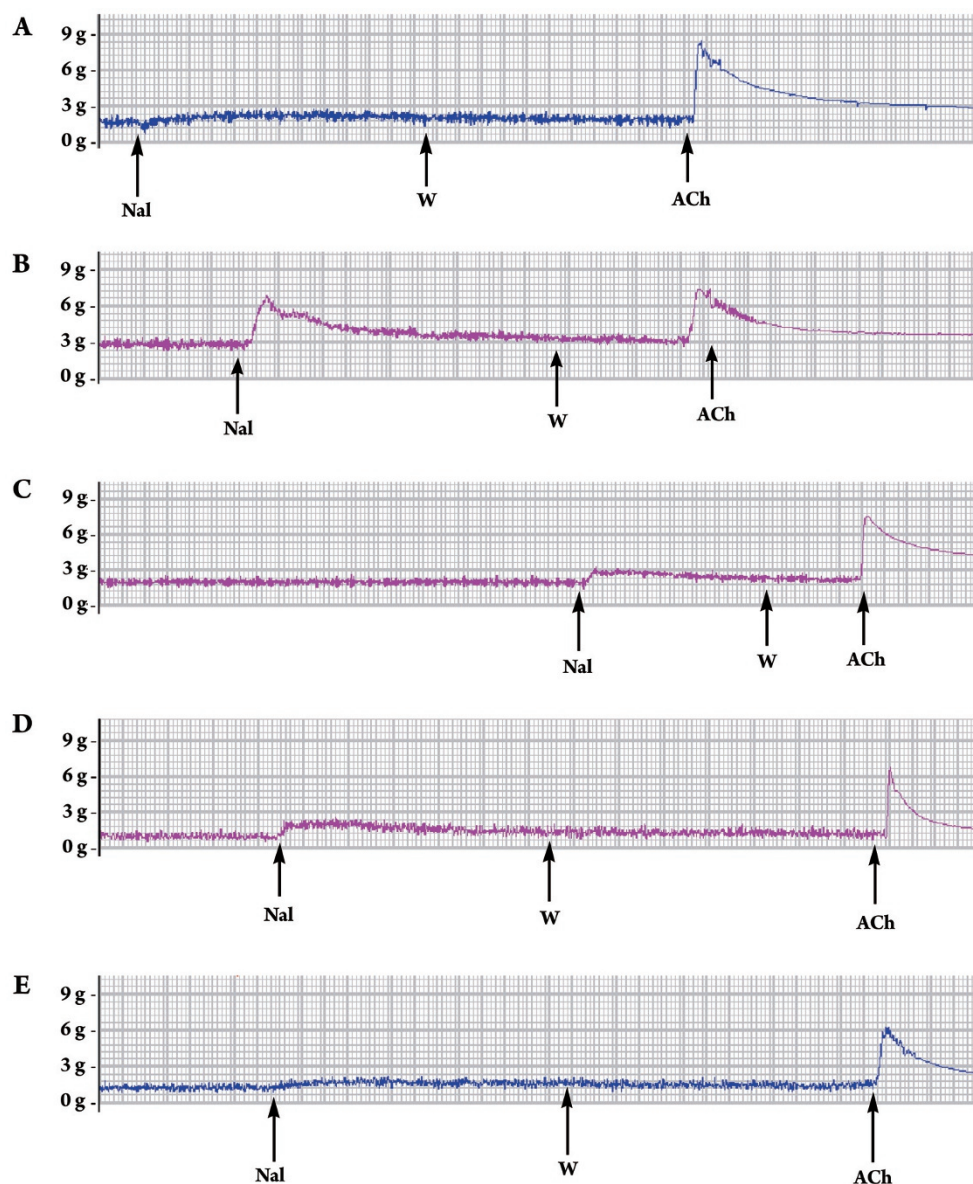
Figure 5. Naloxone (Nal, 3 μmol/L)-induced contraction in the guinea pig ileum incubated with different drugs at 37 °C for 4 h. (A) Vehicle. (B) Codeine phosphate (3 μmol/L). (C) Cholic acid (3 μmol/L). (D) Verticinone (3 μmol/L). (E) CA-Ver (3 μmol/L). W: Wash. ACh: Acetylcholine 3 μmol/L. Withdrawal responses were expressed as the ratio of the ACh 3 μmol/L responses. Vehicle: purified water. Other drugs were dissolved in purified water.

stimulating C-fiber endings and also by stimulating some rapidly adapting receptors with myelinated fibers[26]. Different studies[27, 28] have demonstrated that capsaicin is safe to use and produces a dose-dependent and reproducible cough response in a variety of conditions. Therefore, testing on a capsaicin-induced cough model in mice pretreated with naloxone, a non-selective opioid receptor antagonist, was done to investigate CA-Ver's central antitussive mechanism. The study demonstrated that *po* administration of CA-Ver produced a dose-dependent antitussive effect in mice. The antitussive effect of CA-Ver was significantly reduced by pretreatment with naloxone. In a parallel study, codeine phosphate, one of the most commonly used potent

antitussive agents[29], was used as the positive control. The antitussive effect of codeine phosphate, a centrally acting, narcotic antitussive drug, was also significantly reduced by pretreatment with naloxone. These results indicate that the naloxone-sensitive opioid receptor plays an important role in mediating the antitussive effect of CA-Ver. In our previous study, we had elucidated that the antitussive mechanism of the total alkaloids from *F hupehensis* Hsiao *et* KC Hsia was central through the experimental cough model elicited in pentobarbital-anesthetized cats by electrical stimulation of the superior laryngeal nerve, which had been used to determine the site of the antitussive drugs[30]. Moreover, we had assayed the influences that CA-Ver had on the five kinds of

monoamine neurotransmitters in mouse brain by the reverse-phase HPLC method with a fluorescent light detector, using codeine and dextromethorphan as the positive drugs. All the results indicated that the antitussive mechanism of CA-Ver was related to central opioid receptors and had no relationship with serotonergic mechanisms, just as with codeine.

However, most centrally acting antitussive drugs can lead to the development of tolerance and physical and mental dependence, characteristics that limit the clinical usefulness of these agents. Codeine, a centrally acting (μ_2-opioid receptor agonist), narcotic antitussive drug, was one of the most commonly used potent antitussive agents; however, the addictive nature of codeine limits its clinical usefulness[9]. A physical dependence constitutes an abnormal physiological state produced by the repeated administration of a drug that leads to the appearance of specific symptoms reflective of autonomic, somatic, and behavioral hyperactivity when the drug administration is reduced or discontinued. The overall intensity of the physical dependence is gauged by the severity of the withdrawal syndrome. In order to determine whether CA-Ver is addictive like codeine, we conducted naloxone-urged withdrawal tests of mice. The results indicated that CA-Ver was not addictive.

The isolated guinea pig ileum has been proved to be a model for the study of opioid dependence[19], as it contains populations of functional μ- and κ-opioid receptors[31, 32]. A contractile response was elicited when naloxone was administered to the naive ileum incubated with opioids. These withdrawal responses have pharmacological characteristics similar to the withdrawal signs in the intact animal. So the naloxone-precipitated withdrawal contracture in the ileum has been used as an indication of opioid dependence, since their actions in the enteric nervous system are thought to mimic those in the central nervous system. In the present study, when the ileum was incubated in Krebs solution containing CA-Ver for 4 h, the addition of naloxone did not induce a withdrawal contracture. However, a withdrawal contracture was induced by the addition of naloxone to the ileum treated with codeine phosphate. Taken together with the naloxone-urged withdrawal tests of mice, our results indicated that CA-Ver induced no addiction.

Our present study indicated that the potent antitussive effect of CA-Ver was through the opioid receptor. In both the intact mouse model and the isolated guinea pig ileum, CA-Ver showed no addictive properties. However, opioids such as morphine and codeine also work through the opioid receptor, and the addiction to opioids limits the clinical usefulness of these agents. Do our results contradict these facts? Recent investigations have demonstrated that an antinoci-

ceptive response can be mediated at both supraspinal and spinal sites by μ-, δ-, and κ-opioid receptors[33]. Therefore, not only the μ-opioid receptor but also the δ- and κ-opioid receptors may play an important role in the antitussive effects of opioids. According to reports in the literature, the antitussive effects of opioids are mediated predominantly by μ- and κ-opioid receptors, and some literature indicated that the μ_1-, rather than the μ_1-, opioid receptors were involved in μ-opioid receptor-mediated antitussive effects. However, the addiction of opioids is mediated predominantly by the μ-opioid receptors, not the κ-opioid receptors. Therefore, codeine (a μ_2-opioid receptor agonist) is a narcotic antitussive drug. However, it is not certain whether CA-Ver is a selective κ-opioid receptor agonist or both a κ-opioid receptor agonist and a δ-opioid receptor agonist. The lack of addiction to CA-Ver suggests that the potent antitussive effect of CA-Ver is possibly through the κ-opioid receptor.

Due to the potent antitussive activity but low toxicity of CA-Ver, we investigated its addictive characteristics. This study allowed us to conclude that CA-Ver deserved attention for its potent antitussive effects related to the central opioid receptors without addiction, and that it had a good potential for development as a new antitussive drug.

Author contribution

Jiu-liang ZHANG designed research; Hui WANG and Chang CHEN performed research; Hui-fang PI, Han-li RUAN, Peng ZHANG and Ji-zhou WU contributed analytical tools and analytical support; Jiu-liang ZHANG wrote the paper.

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