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Behavioural and Neurochemical Assessment of Heantos 4 on Preclinical Models of Morphine-Dependence

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Abstract

Objective: Opiate addiction is characterized by compulsive drug use and severe withdrawal symptoms during abstinence. Heantos 4, an herbal pharmacotherapy recently approved for opiate withdrawal treatment in Vietnam, has shown anti-craving properties. The present study is the first preclinical assessment of the effects of Heantos 4 on opiate withdrawal and the rewarding properties of morphine, along with its action on a critical neural substrate of addiction, the mesolimbic dopamine (DA) system.

Methods: Rats received morphine treatments (10 mg/kg, i.p.) for seven days. On Day 8, Heantos 4 (100, 250 or 500 mg/kg, p.o.) was administered prior to naloxone (1 or 10 mg/kg, i.p.). Affective withdrawal symptoms were measured using conditioned place aversion (CPA), and somatic withdrawal symptoms were scored separately. The effect of Heantos 4 on morphine-induced (5 mg/kg, i.p.) conditioned place preference (CPP) was assessed by administering it prior to conditioning, expression or morphine-induced reinstatement. Additionally, the effect of Heantos 4 on the long-term maintenance of morphine-induced CPP was assessed bi-weekly for 6 weeks. Microdialysis studies assessed DA efflux in the nucleus accumbens of rats receiving one or seven repeated treatments of Heantos 4 (500 mg/kg, p.o.) and morphine (10 mg/kg, i.p.), or receiving Heantos 4 and naloxone (10 mg/kg, i.p.).

Results: Heantos 4 reduced somatic but not affective components of naloxone-precipitated opiate withdrawal. It attenuated acquisition but not expression or reinstatement of morphine-induced CPP. Long-term maintenance of morphine-induced CPP was also reduced. Heantos 4 by itself enhanced DA efflux but blunted morphine-evoked DA release on Day 1 and 7. Heantos 4 attenuated naloxone-induced decrease in DA in morphine-dependent rats.

Conclusion: These findings demonstrate that Heantos 4 alleviates symptoms of somatic opiate withdrawal and indicate potential effects on incentive motivation. Moreover, Heantos 4 may modulate DA transmission that limits or antagonizes non-physiological fluctuations in mesolimbic DA activity induced by morphine and naloxone.

Keywords: Heantos 4; Naloxone-precipitated withdrawal; Conditioned place preference; Conditioned place aversion; Morphine; Dopamine; Nucleus accumbens; Microdialysis

Introduction

Dependence on opiates has long been recognized as a significant public health crisis in most regions of the world. In the USA alone, the National Survey on Drug Use and Health [1] reported that 467,000 persons used heroin on a regular basis, whereas prescription opiates were misused by 2.1 million individuals. Most strategies for opiate detoxification treatment rely on opioid substitution such as methadone or buprenorphine which present abuse liability even when coupled with a μ-receptor antagonist [2,3]. This issue highlights the urgency for a new effective treatment with no abuse liability. Recently, the use of Asian medicinal plants has gained attention as clinical and pre-clinical data support their efficacy in alleviating withdrawal and promoting recovery of opiate addiction [4-6]. Moreover, several studies report that the consumption of these plant extracts is not associated with abuse liability [5,7-9].

Heantos 4 is a new pharmacotherapy derived from Vietnamese medicinal plants shown to facilitate opiate detoxification and recovery [6,10]. The formulation of Heantos 4, which conforms to GMP standards, consists of extracts from twelve plants (see Table 1). Recent phytochemical analyses of Heantos 4 detected the presence of 194 identifiable compounds [11-14], some of which are listed in Table 2. Following a successful clinical trial process, Heantos 4 did not present any toxic or adverse side effects and was recently approved by the Vietnamese Food and Drug Administration for in-clinic treatment of opiate addicts undergoing withdrawal from extended opiate dependence [6].

In the absence of peer-reviewed preclinical data concerning the neuropharmacological and behavioural properties of Heantos 4 in the context of addiction to opiate drugs, our laboratory undertook a formal neuropsychopharmacological assessment of this novel medicinal formulation. The first series of experiments assessed the

effect of Heantos 4 on the somatic and affective withdrawal symptoms precipitated by naloxone in morphine-dependent animals. We then investigated the potential abuse liability of Heantos 4 using a conditioned place preference CPP procedure [15-17].

Plant part, genus and species
Radix Campanumoeae
Radix *Ophiopogonis japonici*
Radix *Astragali membranacei*
Radix Glycyrrhizae
Radix *Angelicae sinensis*
Radix *Rehmanniae glutinosae*
Radix Polygalae
Tuber *Stephaniae glabrae*
Rhizoma Zingiberis
Ramulus Cinnamomi
Fructus *Ziziphi jujubae*
Semen *Ziziphi mauritianae*
Binding Agent
Colla Corii asini

Table 1: Plants and binding agent used in the formulation of Heantos 4. Radix=root, rhizoma=tuber; ramulus=twig; fructus=fruit; semen=seed.

This experimental approach was also employed to assess the effect of Heantos 4 on the rewarding and motivational properties of morphine. During the CPP conditioning phase, animals learned to associate a contextual environment with the rewarding effect of a drug. This Pavlovian conditioning effect is long lasting and can be expressed repeatedly for up to 12 weeks [18,19]. CPP can also be used to assess motivation to reinstate drug-seeking behavior after extinction training [18,20,21]. Thus, the present study also examined the effect of Heantos 4 on acquisition, expression, reinstatement and long term maintenance of morphine-induced CPP.

Given the evidence linking activity within the mesocorticolimbic dopamine (DA) system, in part to the reward property of opiates [22-24], a second series of studies assessed the effect of Heantos 4 on DA efflux in the rat nucleus accumbens (NAc) using microdialysis. We examined the effect of Heantos 4 on increased DA efflux induced by morphine as well as decreased DA efflux induced by naloxone in morphine-dependent rats [25,26].

Compound Family	**Phytochemical Name**
Homoisoflavonoids	Methylophiopogonanone A
	Ophiopogonanone A
	Methylophiopogonanone B
Sugars	Ethyl-fructofuranose
	Methy-lfructofuranose
	n-Butyl-fructofuranose
Iridoids and iridoid glycosides	Catalpol
	Ajugol
	Ajugoside
	Rehmaglutin D
	6-O-E-feruloyl ajugol
Nucleotides (amino acids)	Adenosine
	Uridine
	Tryptophan
Flavonoids	5,7-Dimethoxy-3,4-methylenedioxy-flavan-3-ol
	Rutine
Triterpenes	Oleanolic acid
	Betulin
	3α,28-dihydroxyurs-12-ene
	Erythrodiol
Steroids and steroid glycosides	Campesterol
	Stigmasterol
	β-Sitosterol
	Isofucosterol
Tetrahydroprotoberberines	l-Tetrahydropalmatine
	Stepholidine
	(s)-Corydalmine

Table 2: Main phytochemical compounds isolated from Heantos 4 (for a more extensive list, see [11-14]).

Materials and Method

Subjects

Male Sprague-Dawley rats (Charles River, Montreal, Canada) weighing 200-220 g upon arrival were pair-housed in a colony room (21 ± 1°C) under a reverse-light cycle (light off 21 h). Animals that underwent cannulation surgery were subsequently housed individually. Food and water were available ad libitum. All experiments followed the principles of laboratory animal care and were conducted in accordance with the standards of the Canadian Council on Animal Care and the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council 2003) and were approved by the Committee on Animal Care, University of British Columbia.

Drugs

Morphine sulfate (Unipharm Wholesale Drugs Ltd., Richmond, Canada) and naloxone hydrochloride (Sigma-Aldrich, Oakville, Canada) were diluted in 0.9% sterile saline. Heantos 4 was provided by

the Institute of Chemistry of Hanoi in Vietnam in granular form that was insoluble in saline and as such, prepared as a suspension in 0.5% w/v carboxymethylcellulose (Sigma Aldrich, St Louis, USA) in filtered water.

Behavioural experiments

Testing apparatus: The testing apparatus for CPP and CPA (conditioned place aversion) was constructed from acrylic panels. Two larger rectangular compartments (47.2 × 24.6 × 31.5 cm^3) were separated by guillotine doors from a smaller white neutral zone (21 × 16 × 31.5 cm^3) with a smooth Plexiglas floor. The two large compartments had contextually distinct features comprised of different visual and tactile cues. One compartment had black and white striped walls with a wire mesh floor and the other compartment had grey walls with a Plexiglas bar floor. Each apparatus was illuminated by a red light located above the center of the middle compartment. A digital camera placed above each apparatus transmitted data to a computer for analysis with appropriate software (Ethovision, Noldus) providing an accurate measure of time spent in each compartment.

Somatic symptoms of morphine withdrawal: Animals received morphine injections (10 mg/kg, i.p.) for seven consecutive days [26]. On Day 8, animals received vehicle (n=12) or Heantos 4 (100 (n=8), 250 (n=8) or 500 mg/kg (n=8)) by oral gavage 30 min before a naloxone injection (10 mg/kg, i.p.). They were then placed immediately into a rectangular chamber (34.5 × 23.5 × 35.5 cm^3) enclosed with an acrylic top panel for 140 min. The back wall had a mirror reflecting the image of the animal and the front wall was transparent in order to record the withdrawal signs via a camera positioned in front of the chamber. A researcher, blind to the treatment condition, recorded the number of occurrences of writhing (abdominal contractions), wet dog shakes and jumping/hopping as well as the presence or absence of diarrhoea, teeth chattering, swallowing, salivation, chromodacryorrhea, ptosis, abnormal posture, penis grooming and irritability during the first 80 min and between 120-140 min. The intensity of physical withdrawal from opiates was determined using the Gellert-Holtzman scale [27], in which the withdrawal score was calculated by a combination of weighed measure of each individual withdrawal signs and the change in weight of the animals during the session.

Conditioned place aversion: The negative affective state induced by naloxone-precipitated withdrawal in morphine-dependent rats was measured using CPA with a 'single training' and 'single-side conditioning protocol' [28]. On the first day, animals freely explored the CPA apparatus for 20 min and then were randomly assigned to one of the four treatment groups. Starting on the following day, animals received a daily injection of morphine (10 mg/kg, i.p.) for seven consecutive days in the home cage. On the 9th day, rats were orally administered vehicle (n=11) or Heantos 4 (100 (n=8), 250 (n=8), and 500 mg/kg (n=8)) 30 min prior to an injection of a low dose of naloxone (1 mg/kg, s.c.) that was subthreshold for precipitating somatic withdrawal. Immediately after the naloxone injection, the rats were confined to one of the two compartments for 1 h. The assessment of CPA was conducted 72 h after the single conditioning day. Rats were given free access to both compartments for 20 min and the time spent in each compartment was recorded. Previous studies using the single-side protocol showed no difference between a saline-associated compartment and a novel environment [29]. Both environments are considered to be equally preferred and therefore a reliable CPA or CPP effect can be observed using this 'single-side conditioning protocol' [28-30].

Conditioned place preference: On the pre-conditioning day, animals were given 15 min to freely explore the CPP apparatus. Those displaying a strong preference for any of the compartments were eliminated from the experiment. Remaining rats were randomly assigned to one of several treatment groups (specified below). Each rat received the drug of interest or vehicle on alternate days during the 8 day conditioning phase, prior being confined for 45 min to their assigned large compartments [31]. The drug-associated compartment was counterbalanced between the two compartments in a same group treatment. The next day, animals were tested for their preference or aversion to the compartment associated with the treatment drug by placing rats into the middle compartment and then measuring the time spent in each compartment during a 15 min session.

Using this CPP protocol, we first assessed the intrinsic motivational properties of Heantos 4. Heantos 4 (100 (n=7), 250 (n=8), and 500 mg/kg (n=7), p.o.) or vehicle (n=10, p.o.) was administered during the conditioning phase.

In a subsequent study, animals were treated with morphine (10 mg/kg, i.p.) during the conditioning phase. The first experiment assessed the effect of Heantos 4 on the acquisition of morphine-induced CPP by administering this treatment (100 (n=7), 250 (n=10) and 500 mg/kg (n=7), p.o.) or vehicle (n=10, p.o.) 30 min before each of the four pairings of morphine with the drug-associated compartment. A second experiment examined the effect of Heantos 4 on the expression of morphine-induced CPP. Animals were conditioned with morphine or saline and 24 h after the last pairing, received Heantos 4 (100 (n=8), 250 (n=8) and 500 mg/kg (n=10), p.o.) or vehicle (n=13, p.o.) 30 min prior to the test. A third experiment investigated the effect of Heantos 4 on reinstatement of morphine-induced CPP. Animals that acquired and expressed a preference to the morphine-associated compartment during the test day were subjected to an extinction protocol. Animals were exposed daily to the entire apparatus for 15 min until they did not show any preference between the morphine and vehicle-associated compartments. The extinction phase lasted 9 to 11 days. The animals then received Heantos 4 (100 (n=10), 250 (n=9), and 500 mg/kg (n=8), p.o.) or vehicle (n=11, p.o.) 15 min prior to an acute injection of morphine (5 mg/kg, i.p.) and immediately before exploring the entire CPP apparatus for 15 min. Reinstatement was indicated by a significant increase in time in the morphine - compared to the vehicle-associated compartment.

The final behavioural experiment assessed the effect of Heantos 4 on long-term maintenance of morphine-induced CPP. Our previous study showed that morphine-induced CPP persisted for 8 weeks following conditioning [31]. In the present experiment, following confirmation of morphine CPP, rats were assigned randomly to 4 groups (n=6-7 per group), and then administered either vehicle or Heantos 4 (100, 250 or 500 mg/kg; p.o.) in the home cage for 7 consecutive days. Subsequently, these animals were tested for CPP 24 h after the completion of Heantos 4 treatment and then bi-weekly for 6 weeks.

Microdialysis experiments

Surgery: All rats in microdialysis experiments were implanted bilaterally with guide cannulae over the NAc. Rats were anesthetized using 4% isoflurane (Aerrane, Baxter Co., Toronto, Canada) mixed with oxygen, and then maintained at 2.0-2.5% isoflurane for the remainder of the surgery. Rats were given a subcutaneous injection of

an analgesic (ketoprofen 5 mg/kg, s.c.) and then placed in a stereotaxic apparatus in the flat-skull position (mouth bar, -3.2 mm). A heating pad placed underneath the stereotaxic frame helped maintain normal body temperature (37.5ºC). All coordinates were determined using the atlas of Paxinos and Watson [32]. Nitric acid passivated stainless steel guide cannulae (19 gauge × 15 mm) were implanted directly above the NAc (from bregma, +1.7 mm anterio-posterior, ± 1.1 mm medio-lateral; from dura, -1.0 mm dorso-ventral). Guide cannulae were secured in place using four stainless steel skull screws and dental acrylic. Stainless steel obdurators (15 mm) maintained patency of the guides until probe implantation. Rats were allowed to recover from surgery for a minimum of one week prior to participating in an experiment.

Microdialysis: Microdialysis probes were concentric in design, constructed from Filtral 12 AN69HF semipermeable hollow fibers (340 um OD × 2 mm, 65 kD molecular weight cut-off; Hospal, Neurnberg, Germany) and silica inlet-outlet lines (75/150 um ID/OD). Typical in vitro recovery of an external DA standard solution from microdialysis probes was 12% at room temperature (21°C). One day prior to experiments, probes were connected to an Instech liquid swivel (Plymouth Meeting, PA), thoroughly flushed with artificial cerebrospinal fluid (aCSF; in mM: 10 sodium phosphate buffer, 1.2 $CaCl_2$, 3.0 KCl, 1.0 $MgCl_2$, 147 NaCl, pH 7.4) and then inserted into the NAc via the guide cannulae such that the 2 mm membrane spanned -4.8 to -6.8 mm from dura. Flow rate through the probe was maintained at 1 uL/min until the termination of the experiment. Following implantation, animals were placed in a Plexiglas chamber ($40 \times 40 \times 40$ cm^3) with food and water and remained there overnight for ~ 16 h. In the morning, samples were collected at 10 min intervals.

High pressure liquid chromatography: Microdialysis samples were analyzed for DA, using reverse-phase high pressure liquid chromatography (HPLC) and electrochemical detection. The system consisted of an Antec Leyden LC100 HPLC pump (The Netherlands), a Scientific Systems Inc. pulse damper (State College, PA), a Rheodyne 9125i manual injector (20 ul injection loop; Rohnert Park, CA), a Tosoh Bioscience Super ODS TSK column (2 um particle, 2 mm × 10 cm; Montgomeryville, PA) and an Antec Leyden Intro electrochemical detector with a VT-03 flow cell (Vapplied=+650 mV; The Netherlands). The mobile phase (70 mM sodium acetate buffer, 40 mg/L EDTA and 6 mg/L of sodium dodecyl sulfate (variable), pH 4.0, 10% methanol) flowed through the system at 0.17 mL/min. Acquisition and analysis of chromatographic data were accomplished with EZChrome Elite software (Scientific Software, Pleasanton, CA).

Experimental design: The morphine treatment procedure used in microdialysis experiments was the same as that used in behavioural studies of morphine withdrawal symptoms precipitated by naloxone [26]. Animals received either Heantos 4 (500 mg/kg, p.o.) or vehicle 1 h prior to morphine (10 mg/kg, i.p.) for 7 days. On Day 8, rats were treated with either Heantos 4 or vehicle again, and 1 h later, an injection of naloxone (10 mg/kg, i.p.).

Brain microdialysis was used to sample changes in DA efflux in the NAc associated with treatments on Days 1, 7 and 8. Each rat served as a subject on all three days, with sample collection from one hemisphere on Day 1 and from the opposite hemisphere on Days 7 and 8 (i.e., after the conclusion of the experiment on Day 7, probes remained implanted in the NAc for an additional 24 h). Implantation of the probe in the left and right hemispheres was counterbalanced across Days 1 and 7. Each day, HPLC-ED was used to confirm a stable DA baseline (with four consecutive samples showing less than 10% fluctuation in concentration) prior to treating animals with Heantos 4 or vehicle. One hour later, animals were administered a systemic injection of morphine on Days 1 (Heantos 4, n=8; vehicle, n=11) and 7 (Heantos 4, n=7; vehicle, n=13), or naloxone on Day 8 (Heantos 4, n=8; vehicle, n=5). Data from animals experiencing technical difficulties related to either gavaging or HPLC analysis were not included.

Histology: Following microdialysis experiments, rats were deeply anesthetized with isoflurane. Brains were promptly removed and stored in 20% w/v sucrose and 4% v/v paraformaldehyde solution for at least seven days. Brains were then sliced into 50 um coronal sections, stained with cresyl violet and examined for accuracy of probe placement. Only data from those rats with tracts in the shell/core region of the NAc of both hemispheres were included in the present results.

Statistical Analyses

Behavioural data were analyzed using a 2-way analysis of variance (ANOVA) with dose or day as between-subject factors and compartment as the within-subject factor. The critical dependent variable in these experiments was time spent in a morphine-, naloxone- or Heantos 4-associated compartment versus vehicle-associated or non-paired-compartment. Therefore, where appropriate, post hoc analysis (Fisher LSD) was used to show the presence or absence of conditioned place preference or aversion. The opiate withdrawal score was analyzed using a 1-way ANOVA followed by a LSD post hoc analysis. Differences were considered significant if $p<0.05$.

Microdialysis data are presented as % change from the mean value of the final four samples of the baseline period. Basal DA levels were calculated as the mean concentration of the last four baseline samples prior to treatment on each day. Statistical analyses involved 1- or 2-way repeated measures ANOVA, with time as the within-subject factor and treatment (Heantos or vehicle followed by morphine) as the between-group factor. This was followed, when appropriate, by planned comparisons of cell means (Holm-Sidak method), where the significance level for each test was adjusted according to the total number of tests in order to maintain a family-wise rate of Type I error at $p < 0.05$. The Huynh–Feldt correction for non-sphericity was applied to the degrees of freedom for all within-subject analyses. T-tests (one-tailed) were employed for between-group comparisons of peak responses evoked by morphine (Time 120-180 min) in the Heantos- and vehicle-treated groups.

Results

Effect of Heantos 4 on naloxone-precipitated withdrawal

A naloxone injection (10 mg/kg) precipitated somatic symptoms of withdrawal in morphine-dependent animals (Figure 1A). A 1-way ANOVA revealed a significant dose effect of Heantos 4 on the withdrawal score ($F(3,32)=7.3$, $p<0.001$). A subsequent Fisher LSD analysis revealed that rats receiving Heantos 4 at 100, 250 and 500 mg/kg before naloxone had significantly lower scores than vehicle-treated animals ($p<0.01$ or 0.001), indicating that the intensity of somatic withdrawal from morphine was significantly reduced by Heantos 4 pre-treatment.

The affective component of opiate withdrawal was assessed using a low dose of naloxone in a CPA protocol (Figure 1B). A 2-way dose x compartment ANOVA revealed only a significant compartment main effect ($F(1,31)=46.3$; $p<0.001$) and a subsequent Fisher LSD analysis revealed that, in each group treatment, rats spent significantly less time in the naloxone-associated compartment than in the non-paired compartment. These findings indicate that Heantos 4 failed to block or reduce CPA induced by naloxone-precipitated withdrawal in morphine-dependent animals.

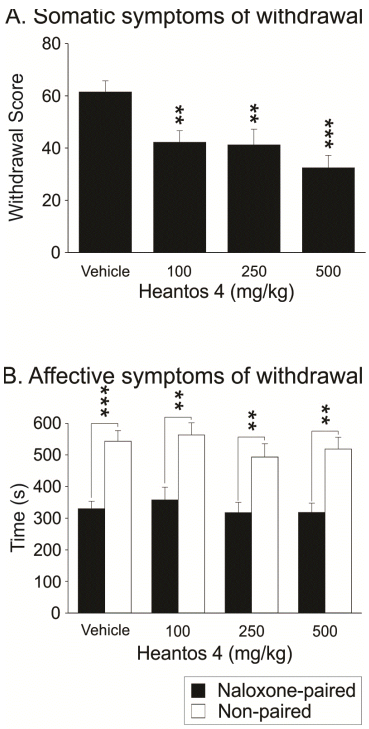

Figure 1: Effect of Heantos 4 on (A) somatic and (B) affective symptoms of withdrawal precipitated by naloxone in rats treated with morphine (10 mg/kg, i.p.) for seven days. (A) Morphine-treated rats received vehicle or Heantos 4 (100, 250 or 500 mg/kg, p.o.) before administration of naloxone (10 mg/kg, i.p.). Data are presented as the mean (+sem) of the total somatic withdrawal score calculated according to the Gellert and Holtzman scale [27]. (B) A test for CPA was conducted 72 hr after the conditioning day where morphine-treated rats received vehicle or Heantos 4 (100, 250 and 500 mg/kg, p.o.) before the administration of naloxone (1 mg/kg, i.p.). Bar graphs represent time spent (mean+sem) in the compartment paired with naloxone and the compartment not paired with any event. Significant difference between (A) vehicle- and Heantos-treated groups or (B) naloxone- and non-paired compartments, $^{**}p<0.01$; $^{***}p<0.001$.

Intrinsic reward properties of Heantos 4

To assess potential intrinsic reward property of Heantos 4, one compartment of the test apparatus was paired with Heantos 4 and the other compartment with vehicle (Figure 2). A 2-way ANOVA showed a significant dose x compartment interaction ($F(3,28)=3.1$; $p<0.05$). A post hoc analysis showed that animals receiving Heantos 4 at 250 and 500 mg/kg doses spent less time in the Heantos 4- compared to the vehicle-paired compartment ($p<0.01$ and $p<0.05$, respectively), indicating that animals exposed to higher doses of Heantos 4 showed an aversion for Heantos 4-paired compartment.

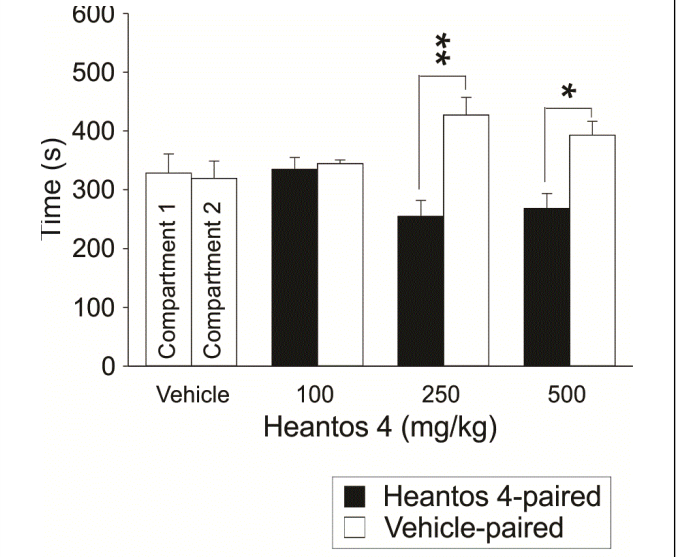

Figure 2: Intrinsic motivational properties of Heantos 4. Bar graphs represent time spent (mean+sem) in Heantos 4- or vehicle-paired compartments during a 15 min test session. Significant difference between morphine- and vehicle-paired compartments. $^{*}p<0.05$; $^{**}p<0.01$.

Effect of Heantos 4 on acquisition, expression and reinstatement of morphine-induced CPP

Administration of Heantos 4 prior to morphine injections during the conditioning period blocked the establishment of morphine-induced CPP (Figure 3A). A 2-way ANOVA revealed a significant dose x compartment interaction ($F(3,30)=3$, $p<0.05$). A subsequent Fisher LSD analysis showed that while animals treated with vehicle and 100 mg/kg of Heantos 4 displayed a preference for the morphine-associated compartment ($p<0.01$ and $p<0.05$ respectively), those that had received higher doses of Heantos 4 (250 and 500 mg/kg) spent comparable amounts of time in both morphine- and vehicle-paired compartments.

When Heantos 4 was administered just prior to the CPP expression test (Figure 3B), a 2-way ANOVA revealed a significant compartment main effect ($F(1,35)=59.2$, $p<0.001$). Post hoc analysis showed that for each treatment group, animals spent significantly more time in the morphine-associated compartment compared to the vehicle-associated compartment ($p<0.05$). Accordingly, Heantos 4 failed to block the expression of morphine CPP.

In the reinstatement experiment (Figure 3C), a three-way ANOVA performed on time spent by each group in the CPP compartments during the expression day and the last extinction day, failed to indicate a significant interaction revealing an absence of significant difference between groups. Therefore, data from the 4 groups of animals were pooled and a subsequent 2-way ANOVA revealed a significant day x compartment interaction ($F(1,37)=23.8$, $p<0.001$; Figure 3C, left

panel). Post hoc tests showed that animals displayed preference for the morphine-associated compartment during the expression day ($p< 0.001$) and had extinguished their preference at the end of the extinction period. During the test for morphine-induced reinstatement, rats received vehicle or Heantos 4 just prior to administration of a morphine challenge (Figure 3C, right panel). A 2-way ANOVA showed only a significant compartment main effect ($F(1,34)=31.5$, $p<0.001$). A subsequent Fisher LSD analysis indicated that at all doses, Heantos 4 treatment failed to affect preference for the morphine- over the vehicle-associated compartment ($p<0.05$ or 0.01), indicating that Heantos 4 failed to block morphine-induced reinstatement of CPP.

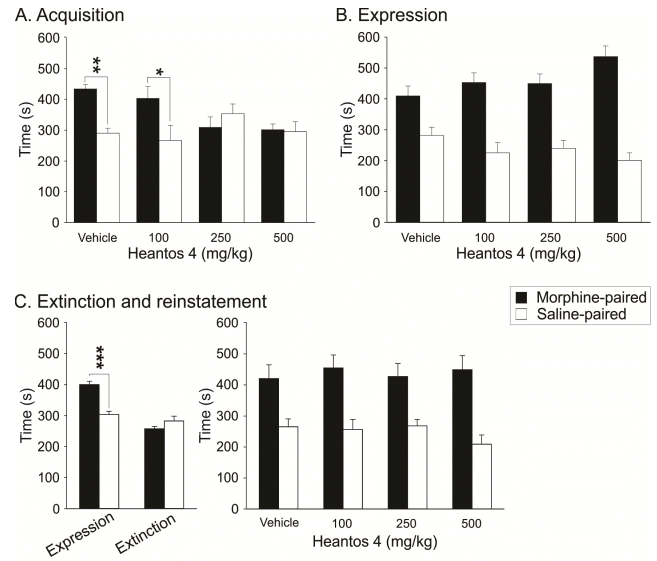

Figure 3: Effect of Heantos 4 on (A) acquisition, (B) expression and (C) reinstatement of morphine-induced CPP. Bar graphs indicate time spent (mean+sem) in the morphine- or vehicle-paired compartments during a 15 min test session. Animals received vehicle or Heantos 4 (100, 250, 500 mg/kg, p.o.) during the (A) conditioning phase and (B) expression day. (C) Extinction followed by reinstatement induced by a priming injection of morphine (5 mg/kg, i.p.). Significant difference between morphine- and vehicle-paired compartments. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

Effect of Heantos 4 on long-term maintenance of morphine-induced CPP

To assess the effect of Heantos 4 on long-term maintenance of morphine CPP, animals were administered a 7 day treatment of Heantos 4 in the home cage immediately after morphine CPP conditioning (Figure 4). A 3-way ANOVA showed a significant dose x day x compartment interaction ($F (12, 88)=1.9$, $p=0.05$), indicating that Heantos 4 affected the long-term maintenance of morphine-induced CPP.

A 2-way ANOVA of time spent in the two compartments by the vehicle group (Figure 4A) revealed a significant day x compartment interaction ($F(4,24)=3$; $p<0.05$). The subsequent Fisher LSD post hoc analysis indicated that vehicle-treated animals spent significantly more time in the morphine- than the vehicle-associated compartments ($p<0.01$ or 0.001), showing that morphine CPP is maintained up to 42 days following conditioning. In Heantos 4-treated animals (Figures 4B-4D), a 2-way ANOVA showed a significant main effect of compartment (100 mg/kg: $F(1,5)=19.8$, $p<0.01$; 250 mg/kg: $F(1,6)=9.5$, $p<0.05$; 500 mg/kg: $F(1,5)=12.9$; $p<0.05$). The day x compartment interaction was non-significant for both Heantos 100 and 250 mg/kg treated animals and the subsequent Fisher LSD analysis revealed that animals treated with Heantos 4 at these doses exhibited a significant preference for the morphine-associated compartment that was maintained until Day 42. However, there was a significant day x compartment interaction ($F(4,20)=2.9$; $p<0.05$) in animals receiving 500 mg/kg of Heantos. Post hoc tests showed that on Day 14 and 42 post-Heantos 4 treatment, these rats spent comparable amounts of time in the morphine- and vehicle-associated compartments, indicating that Heantos 4 at the highest dose decreased the maintenance of morphine CPP.

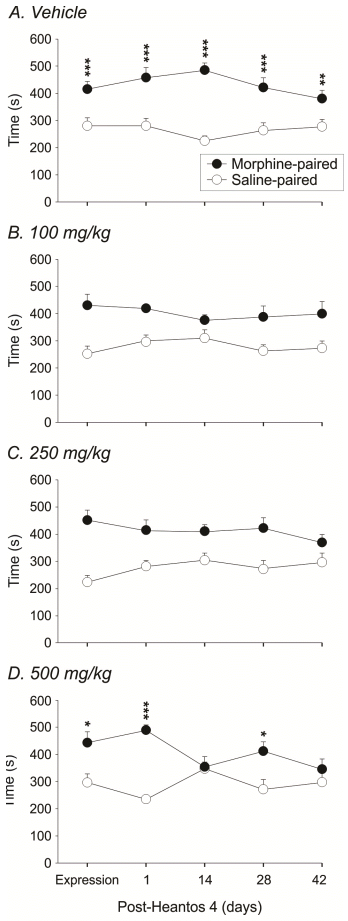

Figure 4: Effect of Heantos 4 on long-term maintenance of morphine CPP. Animals received daily vehicle or Heantos 4 (100, 250, 500 mg/kg, p.o.) for seven days after the morphine CPP conditioning and the test day. Line graphs indicate time spent (mean+sem) in morphine- or vehicle-paired compartments during a 15 min test session on the test day and Day 1, 14, 28, and 42 post-Heantos 4 treatment. Significant difference between morphine- and vehicle-paired compartments. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

Effect of Heantos on morphine-evoked changes in DA efflux in the NAc (Days 1 and 7)

From Days 1 to 7, rats received a daily treatment of Heantos 4 followed 1 h later by morphine (Figures 5A and 5B). On Day 1 (Figure 5A), there was a significant interaction of treatment x time on DA efflux in the NAc ($F(24,240)=17.486$; $p<0.001$). Oral gavage of vehicle was associated with a small brief increase in DA efflux, whereas ingestion of Heantos 4 evoked a significant rise in DA during the initial 60 min sampling period, reaching a peak value of 178 ± 12% above baseline in the dialysis sample taken during Time 30-40 min ($p<0.05$). In the vehicle control group, administration of morphine evoked a significant elevation in DA that reached values greater than 250% from baseline ($p<0.05$). Notably, in comparison to vehicle, pretreatment with Heantos 4 markedly attenuated the morphine-evoked DA response that lasted over 3 h. Peak in morphine-evoked DA response change during the second hour following morphine injection was significantly attenuated in the Heantos 4 condition (Figure 5A, inset, $p<0.05$).

On Day 7 (Figure 5B) a significant interaction of treatment x time on DA efflux in the NAc was observed again ($F(24,144)=4.716$; $p<0.001$). During the pretreatment phase, Heantos 4 again caused a significant rise in DA efflux that peaked at 171 ± 20% (Time 30-40 min, $p<0.05$). Morphine evoked significant increases in DA in all rats, and a between-groups comparison of peak changes (Time 120-180 min) revealed an attenuation of morphine-evoked DA efflux in the Heantos 4 as compared to the vehicle-pre-treated group that approached statistical significance (Figure 5B, inset, $p=0.07$).

Effect of Heantos on naloxone-evoked changes in DA (Day 8)

On Day 8 (Figure 5C), rats treated with morphine for 7 days received either the 8th dose of Heantos 4 or vehicle followed 1 h later by an injection of naloxone. We observed a significant interaction of treatment x time on DA efflux in the NAc ($F(24,264)=3.694$, $p=<0.001$). Naloxone treatment in control animals was associated with an obvious but transient effect on DA efflux. Therefore, statistical comparisons on these data were conducted on DA levels that were averaged across 1 h periods (0-60, 60-120, 120-180, 180-240 min), which revealed a significant effect of naloxone on DA efflux ($F(3,12)=14.465$; $p<0.001$), with a maximal reduction in DA efflux that was approximately 20% below baseline values ($p<0.05$). As observed on Days 1 and 7, treatment with Heantos 4 resulted in a significant increase in DA in all rats, reaching peak levels by 30-40 min (206±23%). Subsequent exposure to naloxone elicited a marked decrease in DA efflux that persisted for 3 h. Importantly, in contrast to the vehicle group, DA efflux in all Heantos 4-treated animals did not fall below baseline values but instead maintained at 20-25% above basal levels. As shown in the inset of Figure 5C, peak change in DA efflux following exposure to Heantos 4 were significantly different from that observed following vehicle pretreatment ($p<0.05$).

Basal DA

On Day 1, prior to any treatments, the basal concentration of DA (uncorrected for probe recovery) in the NAc was not statistically different between rats assigned to the Heantos 4 (1.09 ± 0.23nM) and vehicle groups (0.85 ± 0.11 nM). On Day 7, basal levels were once again comparable between rats that had thus far received six doses of Heantos (0.91± 0.20 nM) or vehicle (0.96 ± 0.17 nM). Following the microdialysis experiment on Day 7, probes remained implanted for an additional 16-17 h. On Day 8, basal DA levels were elevated slightly for rats treated thus far with vehicle (1.60 ± 0.29 nM in the vehicle group) and in those animals receiving Heantos 4 (1.22 ± 0.27 nM); basal values did not differ statistically ($F(6,53)=1.237$, $p=0.302$). However, analyses of basal values based on duration of probe implantation revealed a significant effect on DA concentration ($F(1,58)=5.858$, $p=0.019$). DA concentration was significantly elevated following an implantation period of approximately 40 h (Day 8, 1.37 ± 0.20 nM) compared to 16 h (Days 1 and 7, 0.95 ± 0.09 nM).

Probe placement

The location of microdialysis probe tracts is presented in Figure 5D. The 2 mm lengths of the dialysis membranes were located in the shell/core border of the NAc, spanning a zone 1.2 to 2.2 mm anterior to bregma.

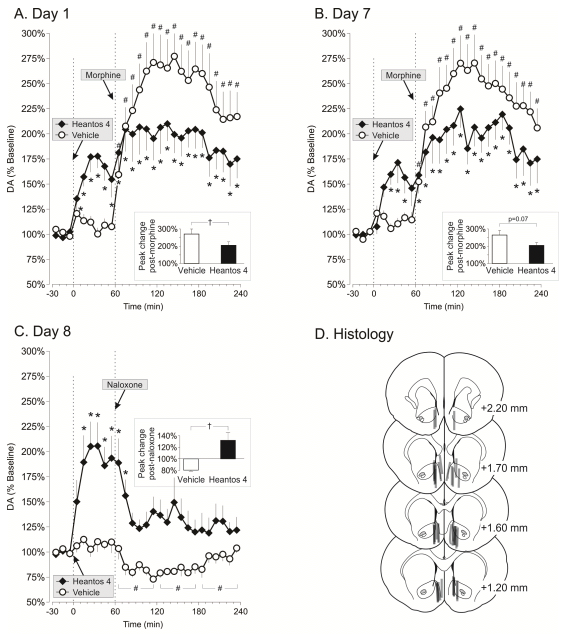

Figure 5: Effect of Heantos 4 on NAc DA efflux on Day 1 and 7 of morphine treatment and naloxone-precipitated withdrawal on Day 8. Rats received Heantos 4 (500 mg/kg, p.o.) and morphine (10 mg/kg, i.p.) on (A) Day 1 and (B) Day 7. Rats gavaged daily with vehicle and injected morphine for 7 days, on (C) Day 8 received vehicles or Heantos 4 (500 mg/kg) and naloxone (10 mg/kg, i.p.). Line graphs represent changes in DA in the NAc (mean+sem). Dashed lines indicate time of Heantos 4 and morphine administration. Bar graphs (A-C) show peak changes in morphine-evoked DA efflux (mean+sem) during Time 120-180 min. $^{*}p<0.05$, comparisons within the Heantos 4 group (vs last baseline value). $^{\#}p<0.05$, comparisons within the vehicle group (vs last baseline value). $^{\dagger}p<0.05$, comparison between the Heantos 4 and vehicle groups. Histological verification of probe placement (D). Vertical lines represent the 2 mm length of dialysis membranes in the NAc of representative rats. Drawings of coronal sections were adapted from Paxinos and Watson [32]. Distance from bregma is indicated.

Discussion

The present study revealed that Heantos 4 decreased the somatic but not the affective signs of naloxone-precipitated withdrawal in morphine-dependent rats. Heantos 4 did not have any intrinsic rewarding properties and in fact, induced aversion at higher doses. Heantos 4 blocked the acquisition, but not the expression or reinstatement of morphine CPP. Interestingly, Heantos 4 decreased the long-term maintenance of morphine CPP. Microdialysis experiments showed that Heantos 4 alone resulted in a robust enhancement of DA efflux in the NAc while significantly reducing the increased DA efflux evoked by an acute or repeated exposure to morphine. Finally, Heantos 4 prevented the decrease in DA efflux below baseline induced by naloxone in morphine-dependent rats.

Effect of Heantos 4 on naloxone-precipitated withdrawal from morphine: affective versus somatic components

Heantos 4 decreased the somatic signs of opiate withdrawal, thus replicating previous observations reported in Vietnamese patients undergoing Heantos 4 therapy [6,10]. However, Heantos 4 treatment did not change the affective component of naloxone-precipitated morphine withdrawal. Previous studies using cfos expression as a measure of neuronal activation report a dissociation in the neural circuitry involved in the affective compared to somatic component of naloxone-induced morphine withdrawal [33]. Our microdialysis experiments confirmed that Heantos 4 blunted the naloxone-induced decrease in NAc DA release. Related cfos studies [33,34] showed that the extended amygdala including NAc is activated during naloxone-induced CPA. However in morphine-dependent animals, lesion studies demonstrated that the dopaminergic system of the central amygdala and not the NAc is crucial in CPA induced by a low dose of naloxone [35,36]. Accordingly, it would appear that Heantos 4 acts preferentially on the neurophysiological system involved in the opiate somatic withdrawal that comprises an extensive neural network including the periaqueducal grey, nucleus tractus solitarius, adrenergic nucleus paragigantocellularis, and hypothalamus [33,37].

Among the compounds identified in Heantos 4, the tetrahydroprotoberberines (THPB) l-THP and SPD [11] are of principal interest given the evidence already linking them to models of addiction. l-SPD is a D1 receptor partial agonist, a D2 receptor antagonist and a 5HT1A receptor partial agonist [38-41]. l-THP is a non-selective D1, D2 and D3 receptors antagonist and also binds to α1 and 2-adrenergic receptors, 5HT1A,D and 5HT4 and 7 other receptors [38,42-47]. Yang et al. [48] reported, in a clinical study on heroin-dependent participants in China, that l-THP administered for one month decreased the somatic symptoms of opiate withdrawal. The antinociceptive effect of both l-THP and l-SPD may provide a partial explanation for the decrease in somatic opiate withdrawal signs [46]. This antinociceptive effect is naloxone - independent and therefore is not mediated by opioid receptors [48]. Instead, it appears to be the result of blocking D2 receptors in dopaminergic corticostriatal pathways and in the arcuate nucleus which in turn increases endorphin release in PAG [46] (see discussion [49]).

Absence of reinforcing properties of Heantos 4 and its effect on the addictive properties of morphine

Our study shows that Heantos 4 does not have any intrinsic reinforcing properties indicating low potential addictive liability. The apparent aversion displayed by animals at higher doses of Heantos 4 has not been reported in humans [6]. This could be a phenomenon related to the use of a rat model, as Heantos 4 has sedative properties which from an evolutionary perspective can be problematic for prey animals.

Heantos 4 treatment during the conditioning phase blocked the acquisition of morphine-induced CPP and there are two possible explanations for this result. First, Heantos 4 may have interfered with the rewarding properties of morphine, which is consistent with the microdialysis results showing that Heantos 4 blunted the morphine-induced DA increase in the NAc. The critical role of the mesolimbic DA system in the acquisition of morphine-induced CPP is consistent with reports that lesions of the dopaminergic system in the NAc [50,51] or administration of a D1 antagonist in the NAc [52] resulted in an attenuation of opiate-induced CPP. The role of DA in the addictive properties of opiates has been challenged in several reviews that question the role of DA as a hedonic marker of the addictive properties of opiates [53,54]. However, it is important to emphasize that the proposed role for DA in opiate and psychostimulant addiction is linked mainly to 'incentive salience' [55-57], as well as aberrant learning of drug addictive behaviour [58,59]. The intrinsic aversive effect of high doses of Heantos 4 counteracting the hedonic properties of morphine must also be considered. However, previous studies [7,60] showing that the two phytochemicals found in Heantos 4, SPD and l-THP did not have any intrinsic aversion or preference and attenuated morphine- and oxycodone-induced CPP, give support for the proposed effect of Heantos 4 on the motivational and learning factors related to the addictive properties of opiates.

Interestingly, the highest dose of Heantos 4 (500 mg/kg) administered daily for one week post-CPP conditioning attenuated the long-term maintenance of morphine CPP. This Heantos 4 administration protocol mirrors the procedure that was used in the clinical study in Vietnam, in which the patients reported a long-term decrease in craving for opioids [6]. The repetitive administration of Heantos 4 post-morphine treatment may reduce the motivation to seek drug required for the maintenance of opioid addiction.

Acute administration of Heantos 4 failed to block the expression and reinstatement of morphine-induced CPP. Perhaps anti-motivational properties develop only after repetitive exposure to Heantos 4. Similarly, repetitive administration of l-SPD during 7 days post-CPP conditioning reduced morphine CPP [7]. This effect has also been reported with l-THP [61,62]. Furthermore, the repetitive administration of l-THP for 6 days post-CPP conditioning reversed the changes in neurobiological measures induced by morphine during CPP conditioning, including increases of dopaminergic and glutamatergic content in the VTA, NAc and PFC, along with changes in the surface expression of receptors (decreased D2 and increased NR2B receptors) in these three brain regions. A similar mechanism might also explain the effect of Heantos 4 observed in the present study.

Heantos 4 and DA release in NAc

Oral ingestion of 500 mg/kg Heantos 4, which is a dose comparable to that prescribed for human opiate addicts, resulted in a robust enhancement of DA efflux in the NAc. Among the constituents of Heantos 4, l-THP and l-SPD may account for this effect. As discussed above, THPBs are D2 antagonists [38,39,63] and also interact with the 5HT1A receptors to modulate DA neuron activity [41,45]. During the initial 60 min of Heantos 4 treatment, we observed a two-fold increase in extracellular levels of DOPAC in the NAc, but no change in 5-HIAA

was detected (data not shown). This elevation of DA and DOPAC levels resembles characteristic neurochemical profiles of typical antipsychotic drugs such as haloperidol, which reduces negative feedback on DA synthesis and release through interaction with the D2 autoreceptor [64-66]. Thus, similar to typical antipsychotics, phytochemicals found in Heantos 4, such as THPBs may act as D2 autoreceptor antagonists to modulate DA transmission.

The present findings confirm earlier observations that systemic morphine, administered acutely or repeatedly, causes a robust increase in DA efflux from mesolimbic DA terminals [26,67-71]. This enhancement of DA synaptic transmission is attributed to the activation of μ-opioid receptors by morphine, specifically on GABA-inhibitory neurons that synapse with DA neurons in the VTA [72]. When activated by endogenous or exogenous opiates, these μ-receptors inhibit the release of GABA, which then releases the inhibition on DA cell firing [73] and subsequently increases terminal DA efflux [74]. Repeated stimulation of the opioid system is believed to trigger compensatory changes of the mesolimbic DA system. Accordingly, we and others have observed in morphine-dependent animals that a hypodopaminergic state is revealed either by discontinuation of μ-opioid receptor stimulation (as would occur during abstinence) or exposure to a μ-receptor antagonist such as naloxone [26,75]. Although prior treatment with Heantos 4 significantly attenuated morphine enhanced DA efflux in both morphine-naive and -dependent animals, Heantos 4 did not prevent the reduction in DA levels elicited by naloxone administration in morphine dependent animals. Nevertheless, it is important to note that extracellular concentration of DA was maintained above baseline levels, effectively avoiding a DA-depleted state. Together, these findings suggest that the modulation of DA activity by Heantos 4 depends on the (compensatory) state of the mesolimbic DA system. This proposed profile of Heantos 4 is reminiscent of a dopaminergic stabilizer [76], in which a DA receptor ligand functions as a partial agonist with the capacity to "counteract both hyperdopaminergic and hypodopaminergic states" [77]. Based on the pharmacological profiles of THPBs mentioned above, we hypothesize that SPD and l-THP in Heantos 4 are the most likely candidates to modulate the effects of both morphine and naloxone on DA activity. Here, a direct interaction between THBPs and the opioid system is unlikely as THBPs do not show affinity for opioid receptors [45].

Benefits of Heantos 4 versus l-THP alone

l-THP is under consideration as a potential treatment for cocaine addiction [8,47] and has also been approved by the Chinese government for use as an anti-nociceptive/sedative agent. However, l-THP at high doses can have adverse side effect such as nausea, dizziness, sleepiness and can cause respiratory inhibition and extra-pyramidal symptoms [47]. In their study on heroin-dependent participants, Yang et al. [48] showed that the group receiving l-THP reported less drug-craving and less relapse than the placebo group. However, they also reported a higher dropout rate from the l-THP group than from the placebo group, which was attributed to adverse side effects of l-THP, possibly related to being a DA receptor antagonist. Moreover, in the 3 months follow up, approximately half of the remaining l-THP group participants and around 85 % of the placebo group relapsed. This report provides a note of caution, while also illustrating the need to improve the efficacy of anti-craving and anti-relapse pharmacotherapy based on the THPB class of compounds. The literature on herbal medicines contains many examples of beneficial synergistic effects of the combination of different herbs when compared to an individual active ingredient. For example, Hong et al. [78] reported that Radix Angelicae Dahuricae slows down the metabolism and degradation of THP. Accordingly, herbal mixture, such as Heantos 4 which contains active ingredients such as l-THP and l-SPD along with other compounds that may enhance the effects of these active ingredients while counteracting potential negative side effects may provide greater therapeutic efficacy. Indeed, an 'open-lable' study performed in Vietnam [6] reported that 90-95 % of the participants did not relapse after 3 months post-detoxification with Heantos 4.

Conclusion

The present preclinical findings show clear positive effects of Heantos 4 on alleviating the somatic symptoms of naloxone-precipitated withdrawal in rats which are encouraging as this could provide a preclinical model for obtaining a better understanding of the mechanisms of action of this potentially very useful and novel therapeutic approach. Moreover, the present data reporting disruption of maintenance of morphine CPP and perhaps even of the acquisition of morphine CPP, provide the first objective preclinical measures of the potential efficacy of Heantos 4 in the control of opiate addiction. Despite these promising results, the need for well-designed and properly conducted double-blind clinical studies with humans seeking assistance from opiate addiction is of paramount importance. Only when such data are available, should Heantos 4 treatment be considered beyond the currently approved facilitation of opiate withdrawal.

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Conflicts of Interest

TVS is a director of HEANTOS, JSC and a beneficial owner of shares in this company. The Institute of Chemistry, Vietnam Academy of Science and Technology holds a patent on Heantos 4 in Vietnam. AGP is listed on a patent for the clinical use of d,l- govadine. SA, BM and CD have nothing to declare.

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