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Institut für Klinische Pharmakologie
Direktor: Prof. Dr. Dr. Gerd Geisslinger

Selective antagonism of opioid-induced ventilatory depression by an ampakine molecule in humans without loss of opioid analgesia

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Lisa Felden

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Dekan:	Prof. Dr. J. M. Pfeilschifter
Referent:	Prof. Dr. med. J. Lötsch
Korreferent	Prof. Dr. Dr. med. K. Zacharowski
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Für meine Eltern

Uli und Maria

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1 Introduction

The use of opioid analgesics is still the most effective way to manage moderate to severe pain in the clinic. Nevertheless, the use of opioid analgesics is associated with the significant risk of respiratory depression. A meta-analysis of 165 papers with data for nearly 20,000 patients found an incidence of ventilatory depression related to opioid administration after surgery of up to 17% [1]. Importantly, fatal outcomes after opioid administration are still observed even under controlled conditions in the clinical setting and in young patients without major pathology [2]. Selective antagonism of the respiratory depressive effects of opioids without decreasing their analgesic effects would be a major step toward opioid safety.

In rats, the ampakine CX717 has been shown to counteract opioid-induced ventilatory depression. When given alone, it did not stimulate ventilation [3-4]. Since CX717 does not interact with the μ-opioid receptor, it does not act as a direct antagonist opposing analgesic opioid effects. Moreover, while nociceptive neurons do express AMPA receptors, administration of ampakines did not affect nociceptive behaviour in laboratory animals [4]. The availability of CX717 for use in humans has now opened the possibility to examine the utility of CX717 as a selective antidote for the prevention of opioid induced ventilatory depression.

The present study was aimed at establishing a proof-of-concept of whether the selective antagonism of opioid induced ventilatory depression by an ampakine translates to humans. We conducted a placebo-controlled, double-blinded cross-over design to assess the effects of pre-administered CX717 on opioid induced ventilatory depression and analgesia in healthy human volunteers.

1.1 Opioid analgesics and respiration depression

1.1.1 Molecular target of the majority of opioid analgesics: μ-opioid receptors

On the molecular level, morphine and other clinically used opioid analgesics act via activation of a group of G-protein coupled receptors, the opioid receptors. Four

different opioid receptor subtypes are known, the μ- (mu), δ- (delta) and κ- (kappa) receptors, and the opioid receptor-like protein (ORL1) [5-6]. Only recently it has been shown that μ-opioid receptor activation is primarily responsible for the wanted analgesic but also the unwanted side-effects, e.g., respiratory depression, of the majority of opioid analgesics. Mu-opioid receptor-knockout mice did not respond to morphine with analgesia, respiratory depression, constipation, physical dependence reward behaviors, or immunosuppression [7]. In contrast, no behavioral responses related to δ- or κ-opioid receptor activation were observed although these receptors were still present in these mice.

Mu-opioid receptors are extensively expressed in pain processing pathways of the whole central nervous system (CNS) including the dorsal horn of the spinal cord, brain stem and sub-cortical and cortical brain regions. Thus, the effects of opioid analgesics can be found on nearly every level of pain transmission and perception [8-9]. The activation of μ-opioid receptors by opioid analgesics leads to the decrease of the release of excitatory neurotransmitters from pre-synaptic neurons into the synaptic cleft and to hyperpolarisation of post-synaptic neurons, resulting in a reduced excitability of nociceptive pathways and brain regions involved in pain perception.

Activation of μ-opioid receptors triggers also numerous unwanted side effects such as e.g., respiratory depression, nausea, miosis and constipation. Some of them can be attributed to the activation of peripheral opioid-receptors (e.g., constipation, urinary retention) [8], but mostly the unwanted side effects can be tracked to opioid sensitive nuclei in the brain stem [10].

1.1.2 Respiration

Respiration is a complex physiological act that is influenced by several mechanisms including chemoreceptors, airway protective reflexes and neurotransmitters. The pre-Bötzinger complex, a small area in the ventrolateral medulla, plays an important role in generating respiratory rhythm together with the retrotrapezoid nucleus/ parafacial respiratory group (pFRG) [11-12]. In vitro experiments showed that the pre-Bötzinger complex was able to maintain an inspiratory rhythm, while the pFRG showed a more

pronounced effect on expiratory activity. Both centers contribute to rhythm generation in vivo, but as breathing in resting conditions consists mainly in active inspiration followed by passive expiration, the rhythm is generated mainly by the pre-Bötzinger complex. A high percentage of the pre-Bötzinger neurons were propriobulbar neurons, i.e., had axonal arborization in the medulla, connecting them to further bulbospinal neurons located e.g., in the ventrolateral reticular formation which served as pre-motoneurons transmitting to spinal respiratory motoneurons. It has been shown that these oscillatory active neurons bared the characteristics of conditional pacemaker neurons, as they responded to depolarization with voltage depending bursts of oscillations. Microinjection of K^+ produced a temporary increase in motor burst frequency while a reduction of the local synaptic transmission with an NMDA-Antagonist reduced the frequency and eliminated the oscillatory output. Isolated lesions in the pre-Bötzinger complex would be first apparent during sleep followed by perturbations during awareness [12], as the influence of the pFRG increases with increasing activity. Animal studies were able to demonstrate, that only the bilateral destruction of the pre-Bötzinger complex lead to ataxic breathing pattern and pathological responses to hypoxia [13] indicating its ability to compensate a unilateral loss. Respiratory rhythm is furthermore a result of the coupled network between pre-inspiratory-neurons (pre-I-neurons) and pre-Bötzinger-complex-neurons [14].

1.1.3 Effects of μ-opioid receptor activation on respiration: respiratory depression

In contrast to pre-I-neurons, neurons responsible for rhythm generation in the pre-Bötzinger complex have been shown to be opiate-sensitive. Slice preparations including only the opioid sensitive pre-Bötzinger complex neurons bathed in the μ-opioid agonist DAMGO showed gradually slowing of respiratory periods which returned to normal after application of naloxone. However, bath application of DAMGO to en bloc preparations of the brainstem, including also pre-I-neurons initially showed a gradual slowing of respiratory periods that abruptly changed to a quantal-slowing pattern, which results from transmission failure of inspiratory bursts to opioid-

depressed pre-Bötzinger-neurons, while the central respiratory rhythm, maintained by pre-I-neurons was not altered [14-15].

1.2 Re-establishing normal respiration during opioid treatment without affecting analgesia

1.2.1 The challenge

The incidence of opioid-induced respiratory depression in a relatively secure environment such as the postoperative setting in the recovery room remains up to 17% [1, 16]. Even under such controlled clinical conditions opioid administration can result in fatal respiratory depression [17-21], requiring a tight medical supervision including measurements of blood oxygenation level and thus needing experienced staff and medical equipment. In critical conditions the respiratory depression must be reversed by administration of the specific antagonist naloxone, making pain management in these situations very difficult, as naloxone not only antagonizes the unwanted side effects, but also the wanted analgesia. The fear of putting patients at a risk of respiratory depression may also lead to an under dosing of opioids and therefore inadequate analgesia. Control of the respiratory effects of opioids without decreasing its analgesia would imply a significant improve for pain therapy as most fatal outcomes under opioid influence can be explained by the unwanted effects on respiration.

1.2.2 Possible targets to selectively prevent opioid induced respiratory depression

Several attempts have already been made to counteract respiratory depression without influencing opioid-induced analgesia. Some of those attempts targeted the Serotonin receptor system, based on the theory, that morphine induces respiratory depression involving the serotoninergic neurons in the brainstem, through a serotoninergic raphe nucleus discharge resulting in a blockade of synaptic inhibition within the pre-Bötzinger complex, leading to a sustained excitation of respiratory neurons and a disturbed respiratory activity. $5HT_{1A}$-agonists presumably act as autoreceptors and inhibit the serotoninergic discharge. Another promising way to

prevent selectively opioid-induced respiratory depression, was targeting $5HT_7$ or $5HT_{4a}$-receptors, as an activation here leads to a G-protein mediated increase in cAMP, and as both receptors could be positively identified in neurons of the pre-Bötzinger complex, also expressing μ-opioid receptors (exerting their effect through a G-protein mediated decrease in cAMP), agonists at those receptors were postulated to directly counteract μ-opioid mediated respiratory depression.

1.2.3 Translational attempts to prevent opioid induced respiratory depression

8-OH-DPAT and buspirone, both $5HT_{1A}$-agonists, were able to restore breathing in rats with opioid-induced respiratory depression [22-23]. An attempt to translate this model from animal research to humans failed, possibly due to an insufficient effect site concentration achievable in humans [24]. Agonists at $5HT_7$ and $5HT_{4a}$–receptors were also examined in this context [25-27]. BIMU8, a $5HT_{4a}$-receptor agonist reestablished respiratory activity within minutes in rats treated previously with fentanyl, without a measurable antagonistic effect on analgesia. Mosapride, the only $5HT_{4a}$-receptor agonist available for the use in humans, failed to counteract respiratory depression in a similar way [28], possibly explained by a lower potency and a worse brain penetration compared to BIMU8.

1.2.4 Activation of AMPA-receptors: A new way to control opioid induced respiratory depression without affecting analgesia

In vitro experiments showed that local perturbation of excitatory aminoacid neurotransmission provoked an increase in respiratory burst frequency and tonic discharge of inspiratory output [29]. Application of neuromodulators, including 5-HT, GABA and μ-opioids affected the respiratory frequency by acting on neurons within the pre-Bötzinger complex. The synaptic interactions between propriobulbar respiratory neurons in the pre-Bötzinger complex involve excitatory amino acids acting on non-NMDA-glutamate receptors, more specifically on the subgroup of AMPA receptors [11]. Blocking this receptor by injection of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), a non-NMDA-receptor-antagonist, completely depletes the respiratory rhythm. Also the phosphorylation of postsynaptic AMPA receptors through cAMP-PKA,

an endogenously active kinase, modulate respiratory currents in pre-Bötzinger-inspiratory neurons [30]. Activation of PKA with forskolin increases the frequency of motor output, while PKA inhibition decreases respiratory frequency. The local application of α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) produced inward currents in the hypoglossus nerve, which serves as a site of measurement for respiratory output and proofs that AMPA receptors are essential for respiratory rhythm generation [31]. Strengthening this theory, bath application of the non-competitive AMPA receptor antagonist 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine (GYKI) completely abolished hypoglossus motoneuron output and blocked respiratory related drives in the pre-Bötzinger complex, while the local application of GYKI to the pre-Bötzinger complex completely blocked respiratory rhythm generation [32]. Contrariwise local application of AMPA increased respiratory burst frequency and depolarized respiratory neurons.

The above mentioned results indicate that the AMPA receptor with its agonists and antagonists plays an important role in rhythm generation, including phosphorylation of the receptor site as a factor modulating respiratory motor output. This implies perhaps a new working point for influencing respiratory depression induced by opioids.

Development of molecules specifically acting on AMPA receptors opens the possibility to counteract the respiratory depressive effects of opioids, without interfering with the desired analgesia. The experimental compound CX717, 1-(benzofurazan-5-ylcarbonyl)morpholine, used in this study acts by allosterically binding to AMPA receptors as agonist, increasing AMPA receptor gated currents at the presence of glutamate, and thus influencing respiratory rhythm generation [4]. Anatomical and *in vitro* studies suggest that while μ-opioid sensitive neurons in the pre-Bötzinger complex are responsible for opioid induced respiratory depression [33], rhythm generation and inspiratory drive transmission can also be influenced by the experimental compound CX717 through involved AMPA receptors. This would hopefully counteract the μ-agonistic effect of opioids on respiration. Differing from other AMPA agonists, which act on the cyclothiazide binding site, the ampakine CX717 binds on another still unidentified binding site and is categorized as “low impact” compound. This novel group of AMPA receptor agonist does not seem to show up-

regulation of trophic factors, such as the brain-derived-neurotrophic-factor (BDNF). As AMPA-receptors are mainly expressed in the central nervous system, a specific agonist is not expected to have a measurable effect at peripheral sites, reducing the likelihood for serious side effects, and respiratory depression could be influenced without interfering with the desired opioid induced analgesia.

In vivo and in vitro experiments showed that the application of CX546, an earlier developed compound, stimulated baseline respiratory frequency and was able to reverse opioid-induced respiratory depression; encouraging the development of further compounds. An animal study with the compound used in this study, CX717, also has already been successful [4]. Respiratory depression in rats induced by the μ-opioid agonist fentanyl responded to CX717, decreasing the respiratory depression without interfering with analgesia.

1.3 Aim of the study

Aim of this study was to proof, whether the selective antagonism of CX717 on respiratory depression could be translated to humans. We therefore used a placebo-controlled; double-blinded cross-over design to assess the effects of CX717 preadministration on alfentanil induced ventilatory depression and analgesia in otherwise healthy human volunteers.

2 Material and Methods

The study protocol complied with the Declaration of Helsinki on Biomedical Research Involving Human Subjects (Somerset West amendment) and was approved by the Ethics Committee of the Medical Faculty of the Goethe-University Frankfurt am Main, Germany.

2.1 Study population

A total of 28 male candidates were screened for the study. Carriers of the μ-opioid receptor mutation N40D were excluded because this might have been a confounder of respiratory depressive opioid effects [34]. Furthermore, subjects in whom capsaicin application during the training session did not produce a decrease in heat pain tolerance by at least 10% ("capsaicin non-responders") were excluded. Two candidates withdrew their written informed consent prior to the beginning of the study. Ten candidates failed to meet the inclusion or exclusion criteria, respectively (physical examination: 2; laboratory tests: 2; N40D carriers: 4; capsaicin non-responders: 2)

We enrolled 16 healthy young men (age 26.6 ±4.6 years, body mass index 23.4 ± 2.7 kg/m^2; mean ± standard deviation). It was deemed adequate to restrict subjects to males since the inclusion of women was not expected to provide any further information regarding proof of concept, although there is no indication that CX717 is associated with reproductive toxicity in animal tests [35]. Subjects were recruited via flyers distributed at the campus of the University of Frankfurt am Main. Calculation of sample size was based on previously obtained data [36]. It resulted in 11 subjects needed to establish an antagonism of ventilatory depression by 50 %.

2.2 Study design

This trial was designed as a randomized, double-blind, placebo- and naloxone-controlled, two-way cross-over study. Setup was chosen to assess the effects of the experimental compound CX717, compared to placebo, on opioid-induced respiratory depression. Naloxone served as a positive control and was not blinded. The aim of this

study was to assess, whether CX717 would have an antagonizing effect on opioid-induced respiratory depression without affecting the opioid-intended analgesia.

Each of the subjects were randomized in one of two equal proportioned groups and completed two study periods receiving CX717 and Placebo in the sequence AB or BA, maintaining a washout period of 7 days in between.

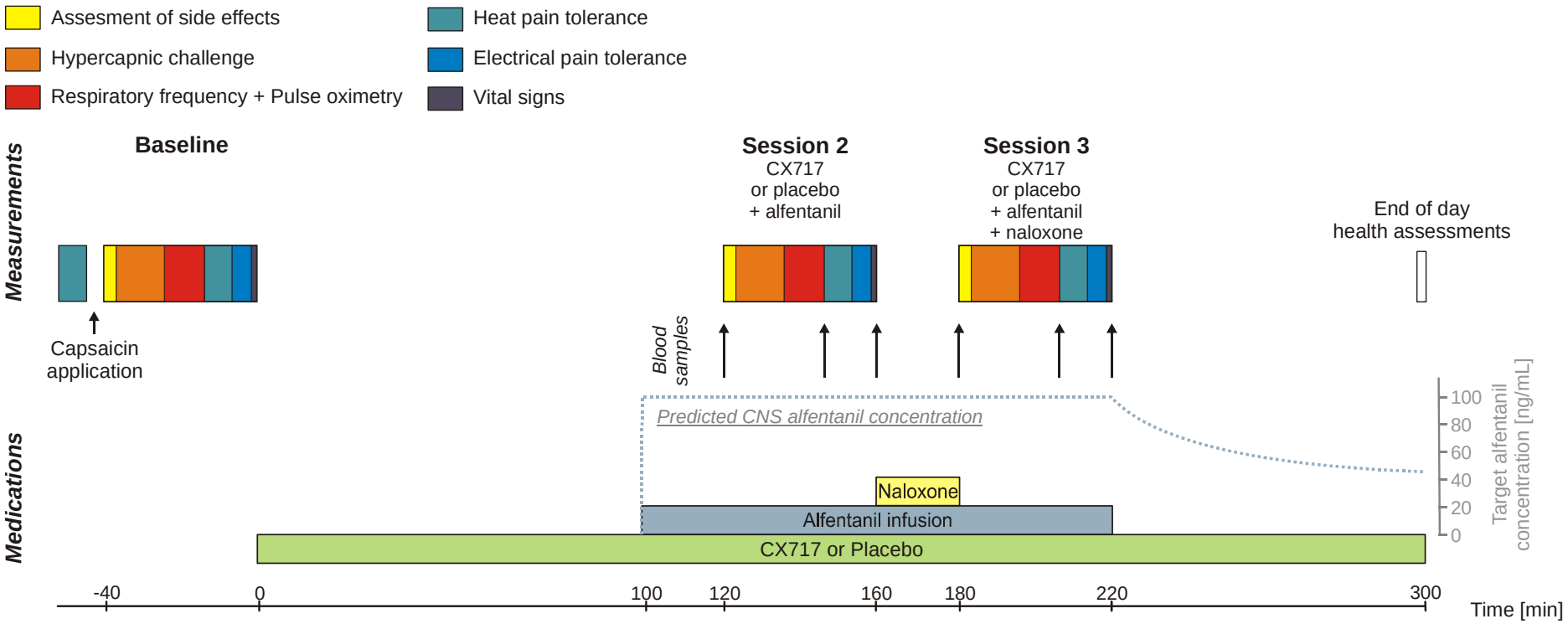
In addition, if a volunteer showed to be eligible for this study a training session was performed to familiarize the subject with the experimental assessments planned to perform during the study periods. Thereafter each subject had to complete two identically designed study periods. After completing the second study period subjects had to attend to a follow-up session in order to assess adverse events or permanent damages.

Each study period comprised a baseline session which meant to assess test responses prior to administering the medication. Subjects were required to fast before each study period. Alcohol was prohibited the 24h prior a study day and any medication was prohibited for 30 days preceding the experiments. A single oral dose of CX717 or placebo was then administered with 240 ml of lukewarm water. Peak concentrations of CX717 were anticipated at approximately 120 min after administration. Thus at both study occasions at 100 min after CX717 administration a computerized intravenous alfentanil infusion was started targeting constant plasma concentrations of 100 ng/ml during the following further assessments for a total of 120 min. During both study periods subjects received alfentanil in an open label fashion and completed a second testing session assessing the effects alfentanil produced on breathing and analgesia starting at 120 min after CX717 administration. By allowing 20 min for equilibration, the effect site concentrations of alfentanil can be considered as equal to plasma concentrations. To evaluate respiratory depression we used two different methods. First, the individual answer to a CO_2-Rebreathing challenge was recorded followed by an additional recording of 6 minutes of spontaneous respiration. Two models were chosen in order to assess whether CX717 had an effect on respiratory rhythm as well as its effect on hypercapnic stimuli. Analgesia was assessed by means of two different peripheral pain models. We chose to assess pain tolerance to transcutaneous electrical

stimulation and to heat pain. Both pain models were equally established in this institute and expected to be able to capture opioid effects as well as the influence of CX717. After assessing both respiratory and analgetic effects for the second time, each subject received a short infusion of naloxone, started at 160 min after administration of CX717 or placebo, intended to antagonize completely the opioid related effects. Immediately following the administration of naloxone, a third assessment session consistent with the previous assessment sessions completed each study period.

The temporal succession of the measurements relative to the administration of the medications is shown below.

Figure 1: Assessment schedule of the CX717 and the placebo condition. Each study condition consisted of three similar sessions, at baseline (Session 1), after initiation of the alfentanil infusion (Session 2) and after the end of the naloxone injection (Session 3). During each session the assessments of ventilatory depression, pain side effects and vital signs was performed. In addition at baseline heat pain values were recorded prior to performing the capsaicin sensitization. Blood sample times and medications are indicated below the assessments, and the anticipated alfentanil plasma (and brain) concentrations are also depicted with a dotted line.



2.3 Study medication

2.3.1 CX717

A single oral dose of 1500 mg of CX717 or placebo was administered after baseline assessment on each study period. Subjects were told to swallow five identically looking tablets together with 240ml of lukewarm tab water provided by the investigator. Study medication was manufactured by the St. Hubertus pharmacy, Berlin, Germany, following randomization codes provided from an external study site. Placebo and tablets containing the experimental compound were identically looking maintaining the double-blind design of the study. Intake was always observed by the investigator.

2.3.2 Alfentanil

In this clinical trial, the participants received in an open-label fashion an intravenous infusion of alfentanil hydrochloride (Rapifen®, JANSSEN-CILAG GmbH, Neuss, Germany). Alfentanil was chosen because of its fast equilibration between plasma and effect site, the central nervous system (CNS), which allows to consider the plasma concentrations as being similar to the brain concentrations after 5 min equilibration time ($t_{½}(k_{e0})$(Alfentanil) = 0.6-1.2 minutes [37]). In addition, the fast equilibration between plasma and brain and the short duration of alfentanil action allowed for keeping the duration of the experiments short and ensured quick recovery of the subjects from the opioid effects after the end of the alfentanil infusion. To maintain a constant alfentanil concentration at the effect site, alfentanil was administered by means of computerized infusion using STANPUMP program ([38] freely available from Steven L. Shafer, MD at http://anesthesia.stanford.edu/pkpd) with Scott's pharmacokinetic parameters for alfentanil (weight adjusted; [37]) and the Harvard pump 22 (Instech Laboratories, Plymouth Meeting, PA, USA) targeting a constant plasma concentration of 100 ng/ml. The alfentanil target concentration of 100 ng/ml has been chosen to ensure measurable ventilatory depression and analgesia [34] without jeopardizing the subjects' health.

2.3.3 Naloxone

Immediately after the second assessment session, at 160 min after administration of the experimental compound CX717, subjects received a total dose of 1.6 mg naloxone (DeltaSelect GmbH, Dreieich, Germany) administered by a constant rate intravenous infusion for 20 min without interrupting the alfentanil infusion. Naloxone was chosen due to its characteristic unselective antagonism of all opioid-related effects.

2.4 Assessment of pharmacodynamic parameters

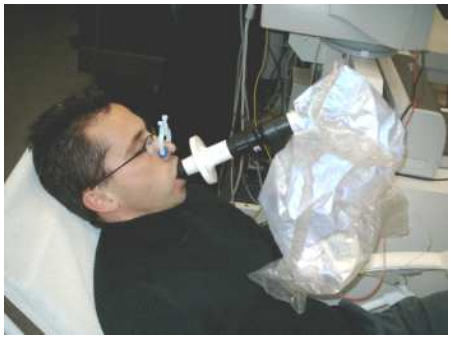
2.4.1 Respiratory depression

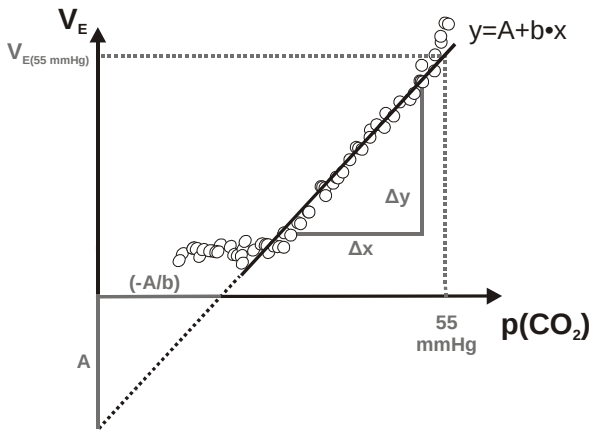
2.4.1.1 Provoking hyperventilation by means of CO_2 re-breathing

Respiratory depression was assessed by means of CO_2 re-breathing. The CO_2 re-breathing was executed according to Read´s method [39] with slight modifications. The subjects were instructed to re-breathe into a plastic bag connected to a special device (Oxycon Pro, Jaeger, Hoechberg, Germany) that continuously recorded the minute expiratory volume ($V_E$min), end-tidal CO_2 levels (pCO2) and ventilatory rate. Being a closed system, the re-breathing leads to an increase of the CO_2 concentration in the plastic bag and as a consequence in the lungs and the body fluids. The progressive rise of the CO_2 concentration in the body fluids due to the continuing metabolism results in an activation of chemoreceptors in the respiratory center. Thus, breathing is further stimulated, finally resulting in hyperventilation, which is indicated by an increase of the minute expiratory volume. To prevent the development of any hypoxic stimulus to the ventilation [39], the plastic bag was filled with oxygen (O_2) prior starting the experiment. The high initial O_2 concentration in the re-breathing bag provided enough O_2 for metabolism, assuring the subjects well-being throughout the assessment. The slope of the linear relationship between the V_E [min] and pCO_2 ($\frac{\Delta y}{\Delta x}$, see Figure 2) is defined as the primary target parameter for the quantification of the respiratory depressive effect. In addition, another reliable parameter is the expiratory volume per minute at a CO_2 concentration of 55 mmHg (VE_{55}), which can be calculated from the slope and intercept of the obtained linear relationship between expiratory volume and

CO_2 concentration [40]. While changes in the x- and y- intercepts can be misleading and therefore should not be used as parameter, a decrease in the slope and VE_{55} can be considered as a reliable indication for respiratory depression during opioid treatment. This method has repeatedly demonstrated to reliably assess the respiratory depressive effects of morphine and alfentanil [28, 41].

Figure 2: Respiratory depression. Assessment of the respiratory depression by means of CO2 re-breathing. Fictitious data acquired during a typical CO2-rebreathing test. The minute expiratory volume (VE) rises with increasing CO2 concentration (dots). Data is analyzed by fitting a linear equation to the data. Respiratory depression is indicated by a decrease of the slope of the linear relationship or a decrease of the minute expiratory volume at a CO2 concentration of 55 mmHg (VE55).



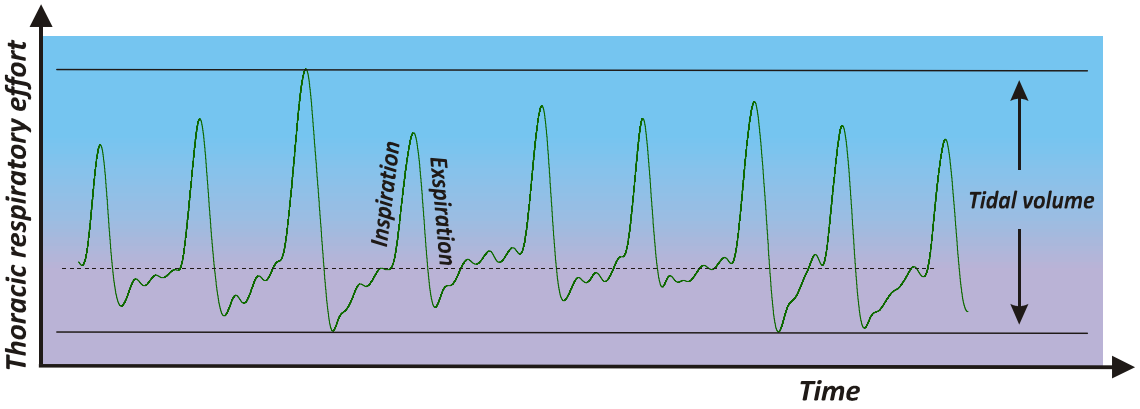


2.4.1.2 Monitoring of spontaneous respiration

Additionally, opioid induced respiratory depression was measured by analyzing changes in the frequency of spontaneous breathing (Figure 3). Therefore, a respiratory belt transducer (MLT1132 Piezo Respiratory Belt transducer, ADInstruments GmbH, Spechbach, Germany) was fastened around the subject's upper abdomen and connected to an integrated data recording unit featuring a built-in amplifier (Powerlab 4/25T, ADInstruments GmbH, Spechbach, Germany). The subjects were instructed to remain silent, while recording their respiratory frequency for a period of 10 min, during which slow music was played and the laboratory was otherwise kept completely silent. The first three and the last minute were rejected from analysis, in order to allow

the subject to adept to testing atmosphere and to normalize breathing. Respiratory depression is indicated by a decrease in the respiratory frequency (Breaths/minute).

Figure 3 Monitoring of spontaneous respiration: Fictitious data acquired during the experiment. Each peak represents a single breath. Results were presented as number of breaths per minutes



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2.4.2 Assessment of analgesia

2.4.2.1 Heat pain tolerance threshold

Heat stimuli were applied with a 3 x 3 cm thermode placed on the skin of the left volar forearm using a Thermal Sensory Analyzer (Medoc advanced Medical Systems Ltd., Ramat Yishai, Israel). Stimuli Temperature was continuously increased by 0.8°C/s starting at 32°C with a maximum temperature of 52.5°C. Pressing a button by the subject triggered cooling of the thermode by 10°C/s down to 32.5°C subsequently starting the next stimulus sequence after an interstimulus interval of 20-25 s. To increase model sensitivity and avoid reaching the technical maximum of 52.5°C, primary hyperalgesia [42] was induced by applying capsaicin cream (0.2%, manufactured by the local pharmacy of the University Hospital Frankfurt am Main) onto the skin area and occluding it with a plaster.

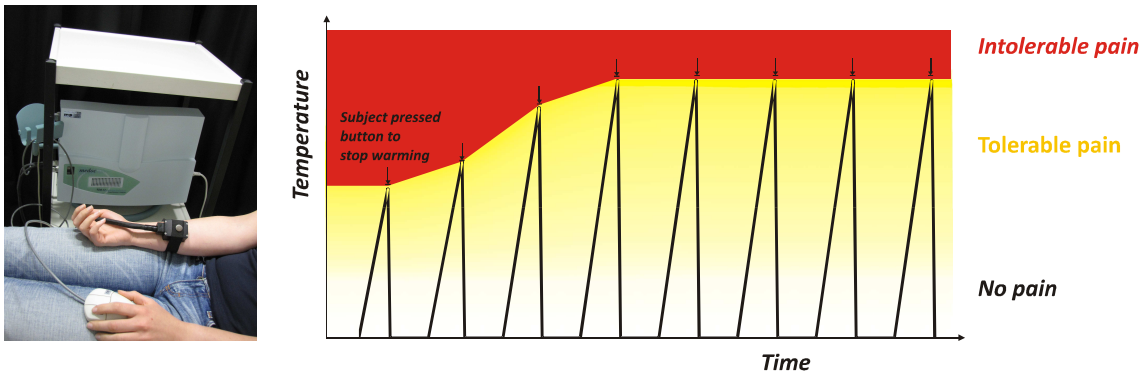
To quantify the individual heat pain tolerance, defined as the change of sensation from painful heat to intolerable heat, heat stimuli started with a temperature of 32.5°C

close to skin temperature and thus known not to be painful. Analgesia was assessed using increasing heat stimuli. Subjects were previously instructed to press a button when the pain reached intolerability in order to stop warming the thermode (Figure 4). After reaching again default temperature of 32.5°C the next stimulus sequence started as described above. A total of 8 heat stimuli were applied in this manner. As previous experiments showed that tolerance temperature reached a plateau after the first three measurements, the median of the last five responses was used as heat pain tolerance.

The analgesic effect of alfentanil is expected to appear as an increase in heat pain tolerance. As the heat stimulus device gave a limit of 52.5°C in order to prevent permanent damaging of the skin, we tried to lower the individual heat pain tolerance with capsaicin. This allowed us to retrieve quantifiable heat pain tolerance values even under the opioid induced analgesia. First heat pain assessment was performed at the very beginning of the study period, followed by application of capsaicin cream (0.2%, manufactured by the local pharmacy) onto the same area the thermode had been placed previously. This area was immediately covered with a plaster for at least 30 min, while performing the other baseline experiments. After removing the plaster, the measurements of heat pain tolerance were repeated exactly in the same way as described above. Capsaicin application to the tested area is expected to lower heat pain tolerance by a minimum of 10%. If any of the subjects would not show this reaction during training session, he would be excluded from further participation in the study and declared as capsaicin non-responder.

In order to prolong the capsaicin effect for as long as study assessments lasted, the tested area was covered with a transparent plaster immediately after each heat pain assessment. After completion of the last testing session capsaicin residuals were removed, and the area was cooled if requested by the subject.

Figure 4: Heat pain model: The subjects received heat pain stimuli of continuously increasing temperature (rate 0.8°C) starting at a temperature of 32.5°C with a maximum of 52.5°C. Temperature increased until the subject stopped heating by pressing a button. Subsequently, cooling of the heating device was triggered until it reached again baseline temperature. The next stimulus was started after an interstimulus interval of 20-25s. The pain threshold was obtained as the median from the last 5 of a total of 8 stimuli applied



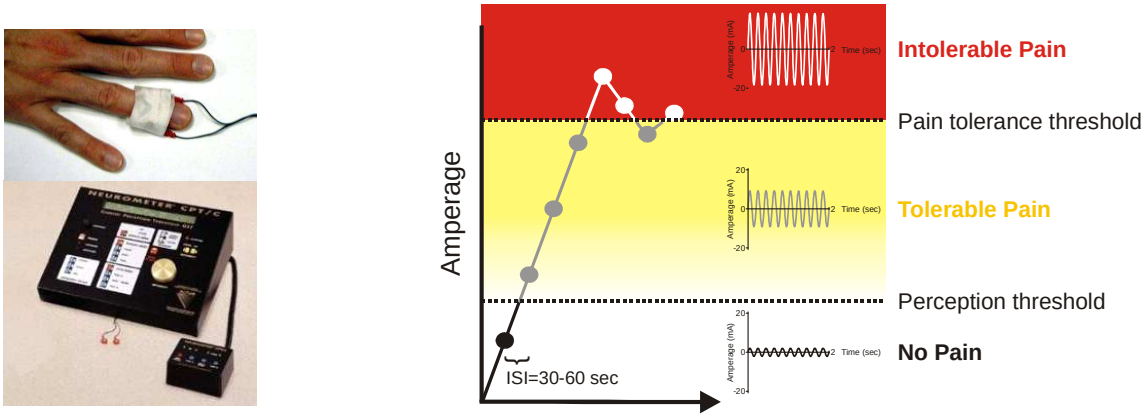
2.4.2.2 Transcutaneous electrical stimulation

Short electrical stimuli (2 s) of increasing intensity were applied to the subjects´ left forefinger (Figure 5). A constant current device (NEUROMETER CPT, Neurotron Inc., Baltimore, MD, USA) with a maximum output of 20 mA was used to administer painful electrical 5Hz sine wave pulses via two gold electrodes placed on the medial and lateral side of the distal phalangeal joint (middle finger of the left hand as default-testing site). The electrical stimulus has been shown to primarily activate C-fibers [43-44] and thus to be able to capture μ-opioid receptor mediated analgesia.

To assess each individuals pain tolerance threshold after electrical stimulation, i.e., the electrical current, when the subjects pain experience changes from tolerable to intolerable, the stimulation started with an electrical current known not to be painful, while starting-stimulus intensity ranged randomly between 0.2 mA and 2 mA. The current was then further increased in an ascending staircase design until the subjects reported to feel pain (Figure 5). The magnitude of subsequent stimuli was determined by the subject's response to the two preceding stimuli. If a subject's response to two preceding stimuli was "no pain – no pain" or "pain – pain" the next stimulus was equal

to the magnitude of the last delivered stimulus plus or minus 130% of the difference between the last and the second last stimulus, respectively. If a subject's response to two preceding stimuli was "no pain – pain" or "pain – no pain" the next stimulus was equal to the magnitude of the second last stimulus plus 75 or 25% of the difference between the last and the second last stimulus, respectively. The purpose of the outlined algorithm was to increase or decrease the magnitude of delivered stimuli quickly as long as a subject gave a uniform response, i.e., "no pain" or "pain". Whenever a subject changed the response from "no pain" to "pain" or from "pain" to "no pain", the outlined algorithm allowed the magnitude of stimuli evoking a change in response at a higher resolution. Subsequently, the pain threshold was obtained from 10 stimuli by logistic regression of "pain" or "no pain" (corresponding to "0" or "1") versus the intensity of the electrical current. The interstimulus interval was approximately 30-60 s. After opioid administration an increase of the pain tolerance threshold is expected, as this pain model has been repeatedly demonstrated to be suitable to quantify the analgesic effects of opioids such as hydromorphone [44], morphine [45], remifentanil [46], or alfentanil [34].

Figure 5: Electrical pain model: The subjects received a 5 Hz sine waves pulses with a length of two seconds in the range from 0-20 mA with an interstimulus interval of approximately 30-60 s. The amount of the electrical current was increased until the subject described the stimulus as painful. Subsequently, the current is decreased until the subject describes the stimulus as non painful. Whenever a subject changes the response from “no pain” to “pain” or from “pain” to “no pain”, the outlined algorithm allows the magnitude of stimuli evoking a change in response at a higher resolution. Subsequently, the pain threshold was obtained from a maximum of 10 stimuli by logistic regression of “pain” or “no pain” (corresponding to “0” or “1”) versus the intensity of the electrical current.



2.5 Assessment of opioid related medical symptoms

Medical symptoms reported by the subjects and the occurrence of vomiting were recorded by the investigator. Opioid related side-effects such as tiredness, nausea, drowsiness and itching were rated by the subject using visual analogue-scales (VAS, length 100mm ranging from 0=”no such symptom” to 100=”symptom experienced at maximum”) at the beginning of each testing session, i.e., at baseline, after equilibration time of alfentanil and after infusion of naloxone.

2.6 Analysis of drug plasma concentrations

To assure that all subjects received similar treatment, drug plasma concentrations were determined by means of liquid chromatographic tandem mass spectrometric (LC-MS/MS). Therefore, venous blood samples (4 ml) were collected into Na-EDTA tubes at predefined time points (n=6 samples for alfentanil and CX717 per study day, n=3 samples for naloxone), with an additional sample taken before administrating any of the medication, serving as blank value. After centrifugation at 3000 rpm for 10 min, plasma was separated, kept on ice until the end of the study day and then frozen at -78°C pending further analysis.

2.6.1 CX717

Aliquots of plasma samples obtained during testing sessions were sent to the US and analyzed at Enthalpy Analytical Inc, Durham, USA. Further information regarding the analyzing process was not available.

2.6.2 Alfentanil

Aliquots of plasma samples were further analyzed to assure similar effect site concentration in all subjects. Alfentanil (Janssen-Cilag, Neuss, Germany) was extracted by protein precipitation using fentanyl (Janssen-Cilag, Neuss, Germany) as internal standard. Therefore, 20 µl human plasma were thoroughly mixed with 20 µl fentanyl-solution (12 ng/ml in acetonitrile), 50 µl acetonitrile and 200 µl methanol. Calibration standards were prepared in the same manner with blank human plasma (Blutspendedienst Hessen, Frankfurt am Main, Germany), but instead of 50 µl acetonitrile, 50 µl of standard solution in acetonitrile was used. The samples were centrifuged for 5 min at 10,000 g and 20 µl of the supernatant was diluted with 200 µl acetonitrile/formic acid (100:0.25, v/v). After another 5 min of centrifugation at 10,000 g, 100 µl of the solution was transferred to glass vials (Macherey-Nagel, Düren, Germany) prior to injection into the LC-MS/MS system. The LC-MS/MS system consisted of an API 4000 triple-mass spectrometer (Applied Biosystems, Darmstadt, Germany) equipped with a Turbo-V-source operating in positive ESI mode, an Agilent 1100 binary HPLC pump and degasser (Agilent, Böblingen, Germany) and a HTC Pal

autosampler (Chromtech, Idstein). For the chromatographic separation an Alltima HP HILIC column and precolumn were used (50 x 2.1 mm I.D., 3 µm particle size from Alltech, Unterhaching, Germany). Mobile phase was acetonitrile/water/formic acid (90:10:0.25, v/v/v), flow rate was 700 µl/min and total run time 2.5 min. Injection volume of samples was 20 µl. Retention time of alfentanil and fentanyl was 1.16 min and 1.03 min, respectively. HPLC-solvents were of HPLC-quality (Merck KgaA, Darmstadt, Germany). Multiple reaction monitoring (MRM) was used for quantification. The mass transitions used were *m/z* 417 → *m/z* 268 for alfentanil and *m/z* 337 → *m/z* 188 for fentanyl. Quantitation was performed with Analyst Software V1.4 (Applied Biosystems, Darmstadt, Germany) using the internal standard method (isotope-dilution mass spectrometry): Ratios of alfentanil and fentanyl peak area were plotted against concentration. Lower limit of quantification of alfentanil was 0.1 ng/ml (14 fg on column). Accuracy of the method over the calibration range (0.1 to 500 ng/ml) was 100.01 ± 5.98 % (n = 8).

2.6.3 Naloxone

Similarly aliquots of human plasma samples were extracted by solid phase extraction and the eluate was analyzed for naloxone. Briefly, 100 µl internal standard containing 30 ng/ml naltrexone and 800 µl water were added to 100 µl plasma. Solid phase extraction was performed on a system consisting of Oasis HLB extraction cartridges (1 ml volume, 30 mg sorbent, Waters, Eschborn, Germany) attached to a Visiprep vacuum manifold (Supelco, Deisenhofen, Germany). The extraction column was activated with 1 ml methanol and 1 ml water. After the sample was drawn through, the column was washed with 1 ml water and dried under a vacuum of -20 mm Hg (-68 kPa) for 7 min. The substances were eluted from the column with 1 ml methanol. The organic solvent was evaporated to dryness by a gentle stream of nitrogen at 40°C. The residue was reconstituted with 100 µl of the mobile phase. Calibration standards were prepared in drug free human plasma and assayed at the beginning of each sequence. For control of inter-assay variation, spiked quality control samples in plasma were measured in each run among samples.

The HPLC equipment consisted of an Agilent 1100 Series binary pump (G1312A) and degasser (G1379A) connected to a HTC PAL autosampler (Chromtech, Idstein, Germany). Chromatographic separations were obtained under gradient conditions using a Synergi Hydro-RP column (150 mm L × 2 mm I.D., 4 µm particle size and 80 Å pore size) (Phenomenex, Aschaffenburg, Germany). The mobile phase consisted of eluent A (water with 5 mM ammonium acetate) and eluent B (methanol with 5 mM ammonium acetate). The gradient was as follows: From t = 0 to t = 0.5 min A/B 99:1, followed from t = 0.5 to t = 7.5 min by a linear gradient from 99:1 to 10:90, then from t = 7.5 to t = 10.5 min 10:90, from t = 10.5 to t = 11 min a linear gradient from 10:90 to 99:1 and finally from t = 11 to t = 14.5 min A/B 99:1. 10 µl of the extracted sample were injected onto the LC-MS/MS. The flow rate was set at 0.3 ml/min and the runtime at 14.5 min. Naloxone and the internal standard naltrexone eluted after 10.5 and 10.3 min, respectively.

MS and MS/MS analyses were performed on a 4000 Q TRAP triple quadrupole mass spectrometer with a Turbo V source (Applied Biosystems, Darmstadt, Germany). For measurement of naloxone and naltrexone the positive ion mode was chosen. High purity nitrogen was used as nebulizer, curtain, auxiliary/turbo heater and collision gas. The heated turbo gas was set at 550 °C. Quantitation was performed in the multiple reaction mode (MRM) using nitrogen as the collision gas with a collision energy of 55 and 39 V for naloxone and naltrexone, respectively. Precursor-to-product ion transitions of m/z 328→212 for naloxone and m/z 342→270 for naltrexone were used for the MRM with a dwell time of 50 ms.

Concentrations of the calibration standards, quality controls and unknowns were evaluated by Analyst software (version 1.4; Applied Biosystems, Darmstadt, Germany). Linearity of the calibration curve was proven from 0.6 to 120 ng/ml. Variations in accuracy and intra-day and inter-day precision (n = 6 for each concentration, respectively) were < 15% over the range of calibration.

2.7 Statistics

Data was submitted to multivariate analysis of variance for repeated measures (rm-ANOVA), with “session” (i.e., baseline, session 2: alfentanil + placebo/CX717, session 3: alfentanil + placebo/CX717 + naloxone; degrees of freedom, df = 2) and “CX717” (i.e., CX717 or placebo; df = 1) as within-subject factors. Pharmacokinetic data were analyzed analogously except fewer degrees of freedom of "session" for alfentanil or naloxone that had not been present at baseline and/or session 2, respectively. Post-hoc comparisons were done by *t*-tests with α-correction (Bonferroni). Effect sizes of CX717 were computed as Cohen's d, which quantifies the standardized difference in parameter means between the effect of interest, percent changes at session 2 as compared to baseline during the CX717 condition, and the placebo effect, percent changes at session 2 as compared to baseline during the placebo condition, calculated as $d = \frac{Mean_1 - Mean_2}{SD_{Combined}}$. The accepted interpretation is a value of d = 0.2 as indicative of a small effect, 0.5 of a medium and 0.8 of a large effect size [47]. Statistics were done with PASW 17.0.2 for Windows (SPSS Inc., Chicago, IL, USA). The α-level was set at 0.05.

3 Results

All 16 subjects completed the study as planned and side effects were mild to moderate. No medical intervention was necessary. Side effects were typical for opioid analgesics and included tiredness, drowsiness, nausea, vomiting and itching.

3.1 Plasma concentrations

The pharmacodynamic results were obtained at similar alfentanil plasma concentrations during the placebo and CX717 conditions (106.0 ± 8.5 ng/ml and 97.4 ± 11.6 ng/ml, respectively; rm-ANOVA factor “session”: F (1,13) = 0.10; rm-ANOVA factor “CX717”: F (1,13) = 4.4, p = 0.06; interaction “session” by “CX717”: F (1,13) = 0.01, p = 0.94; see Figure 6 and Table 1). The naloxone plasma concentrations during session 3 did also not differ between placebo and CX717 conditions (36.1 ± 11.1 and 35.1 ± 15.3 ng/ml, respectively; *t*-test: p = 0.78). CX717 plasma concentrations followed a plateau during the measurements after baseline and were equal during session 2 and 3 (15.4 ± 0.7 mg/ml; rm-ANOVA factor “session”: F (1,15) = 0.007, p = 0.94).

Figure 6: Plasma concentrations of alfentanil, CX717 and naloxone during time-course of the study period

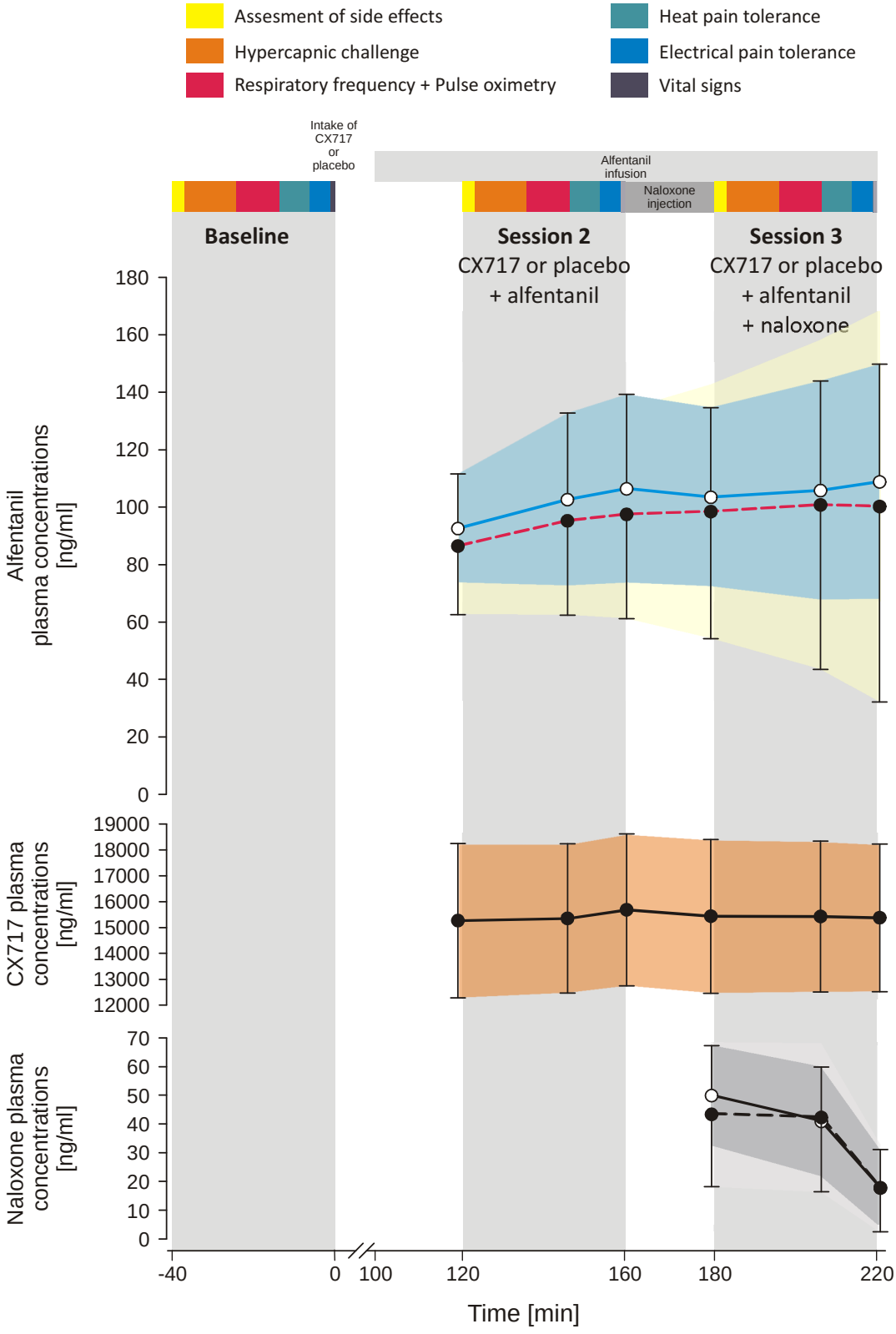


Table 1: Plasma concentrations of alfentanil, CX717, and naloxone during Session 2 and Session 3

		Session 2 (alfentanil)		Session 3 (alfentanil + naloxone)	
Plasma concentrations	N	+ Placebo	+ CX717	+ Placebo	+ CX717
Alfentanil [1] [ng/ml]	14	103.3 ± 27.3	94.8 ± 31.7	108.8 ± 37.4	99.9 ± 56.2
CX717 [2] [mg/ml]	16	-	15.4 ± 2.9	-	15.4 ± 2.9
Naloxone [3] [ng/ml]	16	-	-	36.1 ± 11.1	35.1 ± 15.3

[1] rm-ANOVA: Main effect “CX717”, F(1,13) = 4.4, p = 0.06; main effect “session”, F(1,13) = 0.98, p = 0.34; interaction “CX717” x “session”, F(1,13) = 0.01, p=0.94
[2] rm-ANOVA: Main effect “session” F(1,15) = 0.01, p = 0.94
[3] *t*-test: *t*(15) = 0.28, p = 0.78

3.2 Ventilatory effects

During placebo co-administration, alfentanil decreased the respiratory frequency from 13.1 ± 3.1 min^{-1} at baseline by 25.6 ± 27.9% to 9.2 ± 2.8 min-1 at session 2 (Figure 7 A and Table 2) which was reversed by naloxone (session 3: 12.2 ± 2.5 min^{-1}). During the CX717 condition, alfentanil decreased the respiratory frequency only from 12.2 ± 3.1 min^{-1} at baseline by 2.9 ± 33.4% to 11.1 ± 2.7 min^{-1} at session 2. After naloxone administration, the respiratory frequency returned to 12.4 ± 3 min^{-1} (session 3). The significance of the CX717 effect was revealed by an interaction "session" by "CX717": F (2,28) = 6.8, p = 0.004. In addition, the respiratory frequency differed between sessions (F (2,28) = 5.8, p = 0.008) whereas "CX717" had no significant main effect (F (1,14) = 0.3, p = 0.27) which fits with the similar baseline and post-naloxone measurements during placebo and CX717 conditions. Post-hoc t-tests specified that session 2 (alfentanil) differed significantly from baseline during the placebo ($p < 0.01$) but not the CX717 condition ($p > 0.2$).

The blood oxygen saturation paralleled the observations with respiratory frequency. That is, alfentanil decreased the oxygen saturation from 97.6 ± 0.9% spO_2 at baseline to 94.9 ± 0.9% at session 2, which was reversed by naloxone to 97.2 ± 0.9% (Figure 7 B and Table 2). During CX717 co-administration, the changes were smaller (baseline:

97.6 ± 0.9% spO_2, session 2: 96.2 ± 1.5%, session 3: 97.6 ± 1.1%). The significance of the CX717 effect was again seen as an interaction "session" by "CX717": $F(2,26) = 7$, $p = 0.004$, and the blood oxygenation differed significantly between sessions ($F(2,26) = 33.3$, $p < 0.001$) whereas "CX717" had no significant main effect ($F(1,13) = 3.7$, $p = 0.08$). This fits with the similar baseline and post-naloxone measurements during placebo and CX717 conditions. Post-hoc t-tests specified that session 2 (alfentanil) differed significantly from baseline during the placebo ($p < 0.05$) but not the CX717 condition ($p > 0.2$).

Figure 7: Ventilatory Effects. Opioid effects on spontaneous respiration were attenuated by the ampakine CX717. The respiratory frequency (A) and the blood oxygen saturation (B) decreased with increasing alfentanil concentrations (n = 15 and n = 14, respectively). This was reversed by the naloxone injection. During CX717 co-administration, the opioid induced decrease of the respiratory frequency and blood oxygen saturation was significantly less than under placebo co-administration

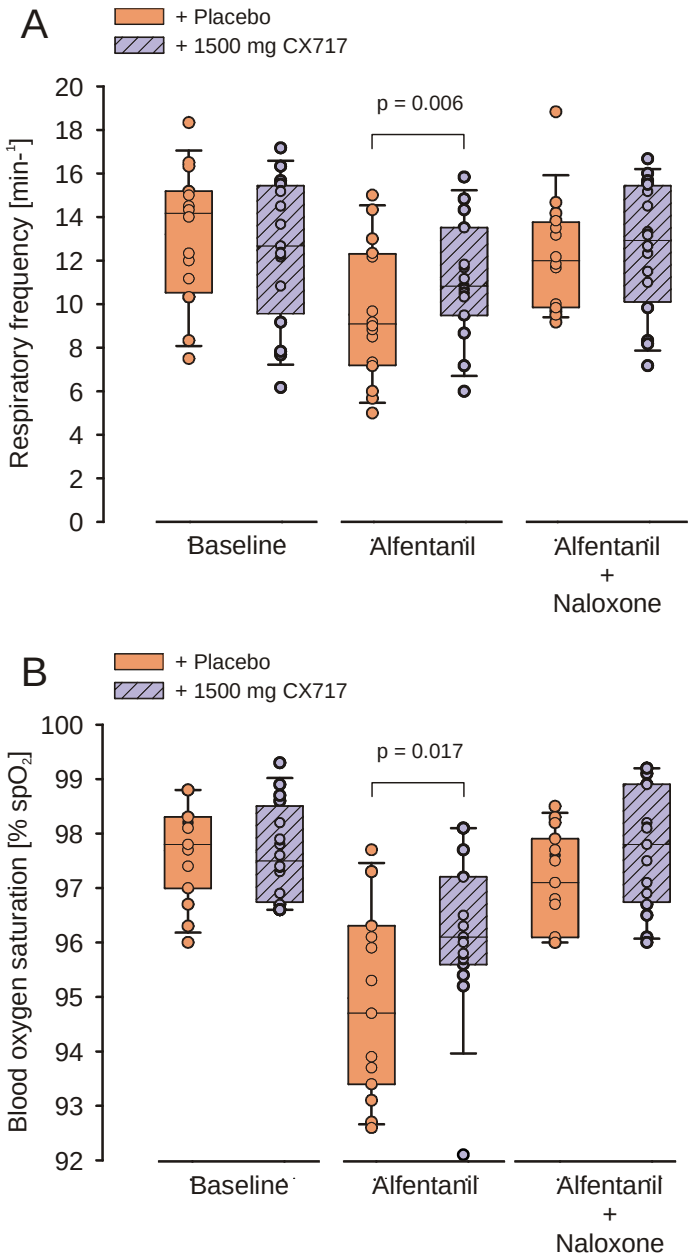


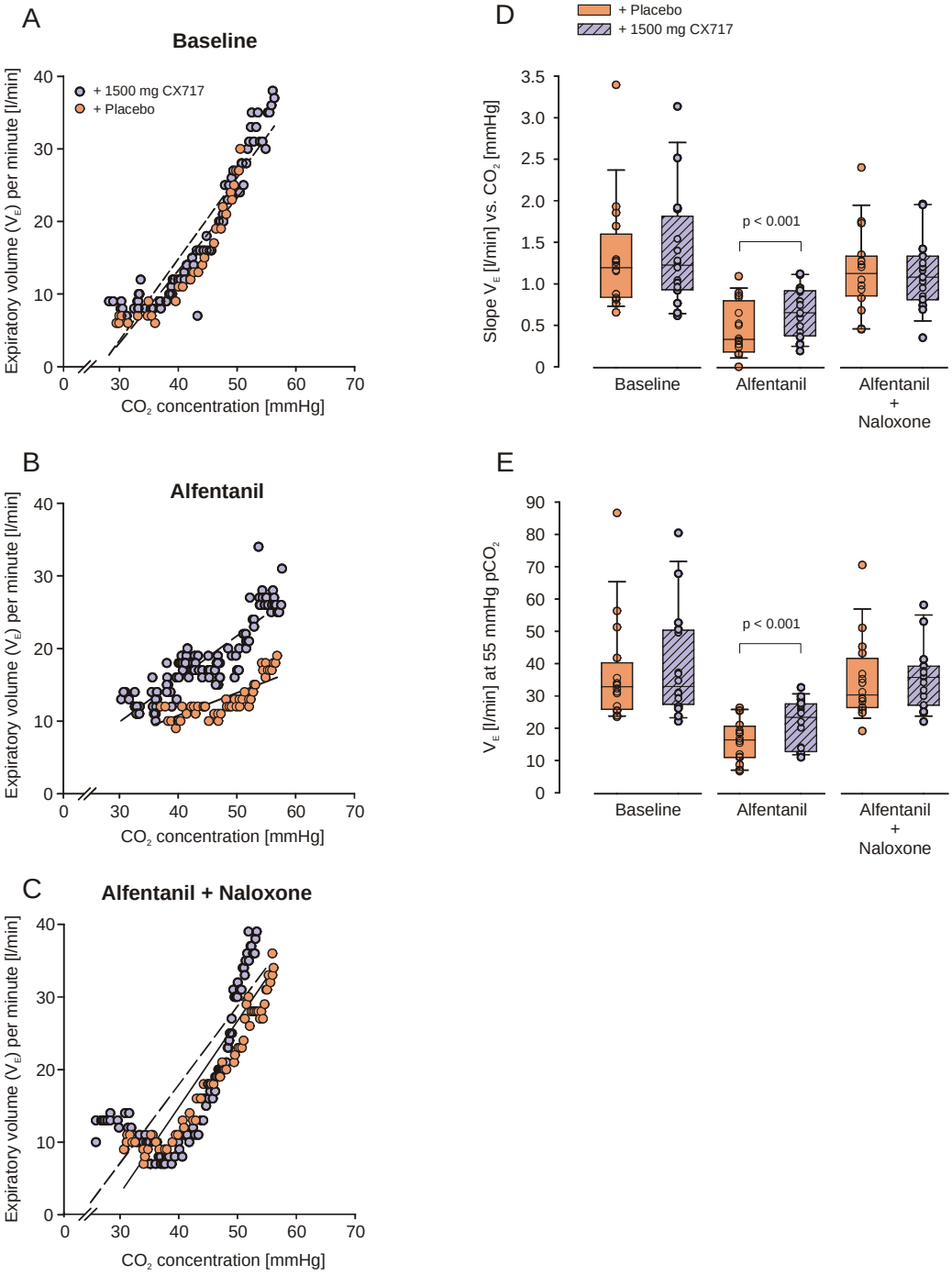
Table 2: Descriptives and statistics of the key parameters of ventilatory depression and analgesia during baseline, session 2 and session 3 (N = number of subjects included in the analysis).

Descriptives								**Statistics**		
		Baseline (pre-dose)		Session 2 (alfentanil)		Session 3 (alfentanil + naloxone)		Main effect "CX717"	Main effect "Session"	Interaction "CX717"x"Session"
Parameter	**N**	**+ Placebo**	**+ CX717**	**+ Placebo**	**+ CX717**	**+ Placebo**	**+ CX717**	**F-value p-value**	**F-value p-value**	**F-value p-value**
Respiratory frequency [min^{-1}]	15	13.1 ± 3.1	12.2 ± 3.3	9.2 ± 2.8	11.1 ± 2.7	12.2 ± 2.5	12.4 ± 3.0	F(1,14) = 0.3 p = 0.27	F (2,28) = 5.8 p = 0.008 [1]	F (2,28) = 6.8 p = 0.004
Hem. oxygenation [% spO_2]	14	97.6 ± 0.9	97.7 ± 0.9	94.9 ± 1.8	96.2 ± 1.5	97.2 ± 0.9	97.6 ± 1.1	F (1,13) = 3.7 p = 0.08	F (2,26) = 33.3 p < 0.001 [2]	F (2,26) = 7.0 p = 0.004
Slope [l · min^{-1} · $mmHg^{-1}$]	15	1.35 ± 0.68	1.43 ± 0.68	0.49 ± 0.31	0.68 ± 0.29	1.21 ± 0.48	1.11 ± 0.44	F (1,14) = 1.7 p = 0.22	F (2,28) = 32.5 p < 0.001 [3]	F (2,28) = 3.8 p = 0.035
VE at 55 mmHg CO_2 [l · min^{-1}]	15	37.9 ± 16.3	40.5 ± 16.5	16.7 ± 6.3	21.9 ± 7.3	35.8 ± 12.4	35.6 ± 10.0	F (1,14) = 6.4 p = 0.024	F (2,28) = 31.5 p < 0.001	F (2,28) = 2.8 p = 0.08
Electrical pain tolerance [mA]	16	4.1 ± 1.1	4.6 ± 1.6	7.0 ± 2.4	7.2 ± 2.6	4.4 ± 1.3	4.0 ± 1.3	F (1,15) = 0.1 p = 0.75	F (2,30) = 38.6 p < 0.001 [4]	F (2,30) = 2.4 p = 0.11
Heat pain tolerance [°C]	16	38.6 ± 4.0	38.1 ± 3.2	48.1 ± 3.9	46.9 ± 3.2	45.4 ± 3.3	44.6 ± 3.5	F (1,15) = 2 p = 0.18	F (2,30) = 154.3 p < 0.001 [5]	F (2,30) = 0.3 p = 0.73

[1] Post-hoc *t*-test: Session 2 differed significantly from baseline during the placebo ($p < 0.001$) but not during the CX717 condition ($p > 0.2$)
[2] Post-hoc *t*-test: Session 2 differed significantly from baseline during the placebo ($p < 0.05$) but not during the CX717 condition ($p > 0.2$)
[3] Post-hoc *t*-test: Session 2 differed significantly from baseline during the placebo ($p < 0.01$) and during the CX717 condition ($p < 0.01$)
[4] Post-hoc *t*-test: Session 2 differed significantly from baseline during both, the placebo and the CX717 condition ($p < 0.001$)
[5] Post-hoc *t*-test: Session 2 differed significantly from baseline during both, the placebo and the CX717 condition ($p < 0.001$)

With hypercapnic challenge during placebo co-administration, alfentanil decreased the slope of the linear relationship between minute expiratory volume and the carbon dioxide concentration in expired air from 1.35 ± 0.68 l min^{-1} ·$mmHg^{-1}$ at baseline to 0.49 ± 0.31 l min^{-1} ·$mmHg^{-}$1 at session 2 (Figure 8 and Table 2). It returned after naloxone administration to 1.21 ± 0.48 l min^{-1} ·$mmHg^{-1}$. During the CX717 condition, alfentanil decreased this slope less (baseline: 1.43 ± 0.68 l·min^{-1} ·$mmHg^{-1}$, session 2: 0.68 ± 0.29 l·min^{-1} ·$mmHg^{-1}$, session 3: 1.11 ± 0.44 l·min^{-1} ·$mmHg^{-1}$). The significance of the CX717 effect was revealed by an interaction "session" by "CX717": $F(2,28) = 3.8$, $p = 0.035$. In addition, the slope differed between sessions ($F(2,28) = 32.5$, $p < 0.001$) whereas "CX717" had no significant main effect ($F(1,14) = 1.7$, $p = 0.22$). However, the reversal by CX717 was incomplete as reflected in significant post-hoc t-tests: session 2 (alfentanil) versus baseline ($p < 0.01$) during both placebo and CX717 conditions ($p < 0.01$). The calculated value of VE_{55} showed a similar tendency as the slope (Figure 8 and Table 2). During placebo co-administration, alfentanil decreased VE_{55} from 37.9 ± 16.3 l min^{-1} at baseline by 53.7 ± 16.6% to 16.7 ± 6.3 l min^{-1} at session 2. It returned after naloxone administration to 35.8 ± 12.4 l min^{-1}. The opioid-associated decrease was smaller (by 40.9 ± 24.3%) during CX717 co-administration (baseline: 40.5 ± 16.5 l min^{-1}, session 2: 21.9 ± 7.3 l·min^{-1}, session 3: 35.6 ± 10 l·min^{-1}). The baseline difference was reflected in a significant main effect of "CX717" ($F(1,14) = 6.4$, $p = 0.024$). "session" had also a significant main effect on VE_{55} ($F(2,28) = 31.5$, $p < 0.001$) whereas the interaction "CX717" by "session" displayed only a tendency toward statistical significance in this parameter ($F(2,28) = 2.8$, $p = 0.08$).

Figure 8: Ventilatory effects. CX717 significantly attenuated the opioid induced hypercapnic response. Representative data from one subject of the relationship between expiratory volume (VE) and CO_2 concentration in the inspired air **(A)** before and **(B)** after administration of alfentanil, and **(C)** after administration of naloxone (median/solid line, mean/dotted line, 5^{th} and 95^{th} percentiles/bars). Opioid effects on all measured respiratory parameters were attenuated by the ampakine CX717. Similarly, **(D)** the slope of the linear relationship between expiratory volume (VE) and CO_2 concentration in the inspired air and **(E)** the calculated expiratory volume per minute at 55 mmHg p CO_2 (VE_{55}) decreased after alfentanil administration (n = 15). Both parameters were also restored by naloxone injection.


A
Baseline
+ 1500 mg CX717
+ Placebo
Expiratory volume (V_E) per minute [l/min]
CO_2 concentration [mmHg]
B
Alfentanil
C
Alfentanil + Naloxone
D
+ Placebo
+ 1500 mg CX717
Slope V_E [l/min] vs. CO_2 [mmHg]
p < 0.001
Baseline
Alfentanil
Alfentanil + Naloxone
E
V_E [l/min] at 55 mmHg pCO_2
p < 0.001
Baseline
Alfentanil
Alfentanil + Naloxone

3.3 Analgesic effects

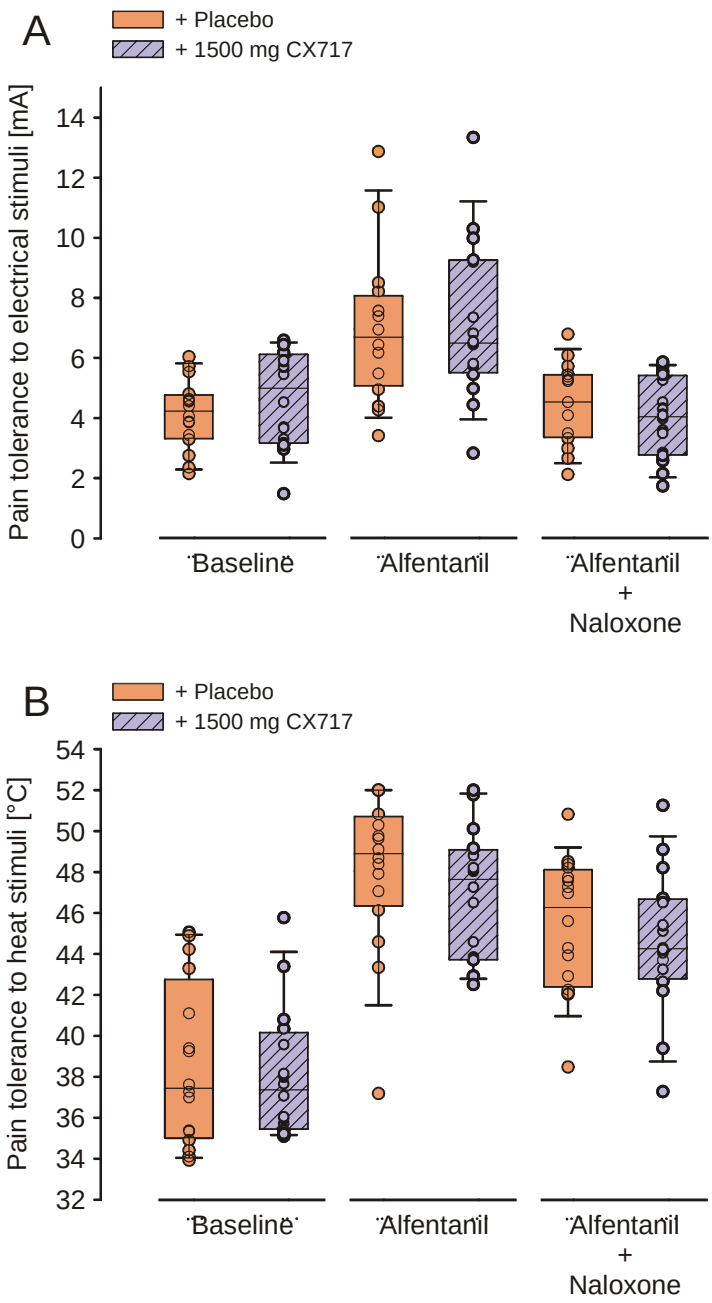
CX717 did not affect opioid induced analgesia. During placebo co-administration alfentanil increased the pain tolerance to electrical stimuli from 4.1 ± 1.1 mA at baseline by 76.4 ± 71.4% to 7 ± 2.4 mA at session 2, which was reversed by naloxone to 4.4 ± 1.3 mA (session 3; Figure 9 A and Table 2). During CX717 co-administration, alfentanil increased the electrical pain tolerance from 4.6 ± 1.1 mA at baseline by 57.6 ± 26.0% to 7.2 ± 2.6 mA at session 2, which was reversed by naloxone to 4 ± 1.3 mA (session 3). The effect of CX717 was not significant as shown by the absent interaction "session" by "CX717": $F(2,30) = 2.4$, $p = 0.11$. In addition, the pain tolerance differed between "sessions" ($F(2,30) = 38.6$, $p < 0.001$) but not "CX717" ($F(1,15) = 0.1$, $p = 0.75$). Post-hoc t-tests specified that session 2 (alfentanil) differed significantly from baseline during both the placebo and CX717 conditions ($p < 0.001$).

CX717 co-administration did also not affect the alfentanil-induced increase in heat pain tolerance. Specifically, during the placebo condition pain tolerance increased from 38.6 ± 4 °C at baseline to 48 ± 3.9°C at session 2 (Figure 9 B and Table 2). It was reduced under the influence of naloxone to 45.4 ± 3.3 °C. Similarly, during the ampakine condition pain tolerance increased from 38.1 ± 3.2 °C at baseline to 46.9 ± 3.2 °C at session 2 and was reduced after naloxone to 44.6 ± 3.5 °C. Again, the effect of CX717 was not significant (interaction "session" by "CX717": $F(2,30) = 0.3$, $p = 0.73$) whereas pain tolerance differed between "sessions" ($F(2,30) = 154.3$, $p < 0.001$) but not "CX717" versus placebo ($F(1,15) = 2$, $p = 0.18$). Post-hoc t-tests specified that session 2 (alfentanil) differed significantly from baseline during both the placebo and CX717 conditions ($p < 0.001$).

The effect sizes of CX717 versus placebo were larger for respiratory depression than for analgesia related parameters. The effects on respiratory depression were medium for respiratory rate ($d = -0.694$) and VE_{55} ($d = -0.65$), and large for the slope of the linear relationship between expiratory volume and the CO_2 concentration in expired air

in the re-breathing experiment (d = -0.8). In contrast, the effects on analgesia were small (electric pain tolerance: d = -0.35, heat pain tolerance: d = -0.2).

Figure 9: Analgesic effects: **(**A) Electrical pain tolerance and (B) heat pain tolerance of capsaicin sensitized skin increased after alfentanil administration (n = 16). The electrical pain tolerance was reversed to baseline levels after naloxone administration. The alfentanil induced increase of heat pain tolerance was significantly but incompletely antagonized by naloxone and did not reach baseline levels. CX717 had no statistically significant influence on the analgesic effects.



3.4 Side effects

CX717 was tolerated by all subjects without the occurrence of side effects requiring medical intervention. Under placebo co-administration, the alfentanil infusion significantly increased tiredness, drowsiness, nausea and itching, which was significantly reduced by naloxone with the exception of nausea that increased immediately after naloxone injection. While CX717 co-administration did not affect drowsiness, nausea and pruritus, it led to a significantly more pronounced increase in tiredness during alfentanil administration (placebo condition: baseline: 19.8 ± 22.8 mm VAS, session 2 (alfentanil): 33.2 ± 28 mm, session 3 (alfentanil + naloxone): 15.3 ± 10.1 mm; CX717 condition: baseline: 8.9 ± 9.7 mm, session 2 (alfentanil): 36.2 ± 30.4 mm, session 3 (alfentanil + naloxone): 13.9 ± 11.6 mm; rm-ANOVA interaction “session” by “CX717”: $F(2,30) = 3.9$, $p = 0.03$; see Table 3). Tiredness also differed between “session” ($F(2,30) = 6.2$, $p = 0.006$) whereas "CX717" alone had no significant main effect ($F(1,15) = 1.6$, $p = 0.23$)

Table 3: Descriptives and statistics of the side effects tiredness, drowsiness, nausea and pruritus during baseline, session 2 and session 3 (N = number of subjects included in the analysis)

		Descriptives						**Statistics**		
		Baseline (pre-dose)		**Session 2 (alfentanil)**		**Session 3 (alfentanil + naloxone)**		**Main effect "CX717"**	**Main effect "Session"**	**Interaction "CX717"x"Session"**
Parameter	N	+Placebo	+CX717	+Placebo	+CX717	+Placebo	+ CX717	F-value p-value	F-value p-value	F-value p-value
Tiredness [mm VAS]	16	19.8 ± 22.8	8.9 ± 9.7	33.2 ± 28	36.2 ± 30.4	15.3 ± 10.1	13.9 ± 11.6	F(1,15)=1.6 p = 0.23	F(2,30)=6.2 p = 0.006	F(2,30)=3.9 p=0.03
Drowsiness [mm VAS]	16	5 ± 13.6	1.2 ± 2.3	46.9 ± 23	43.7 ± 23.2	13.9 ± 17.1	15.2 ± 11.4	F(1,15)=0.4 p = 0.52	F(2,30)=44.4 p = 0.000	F(2,30)=0.41 p=0.67
Nausea [mm VAS]	16	0.3 ± 0.6	0.3 ± 0.5	1.6 ± 3.1	9.4 ± 18.3	10 ± 10.8	15.4 ± 18.1	F(1,15)=4.1 p = 0.06	F(2,30)=8.4 p = 0.001	F(2,30)=1.8 p=0.19
Pruritus [mm VAS]	16	1.4 ± 3.7	0.3 ± 0.4	20.9 ± 22.2	15.4 ± 21.1	0.4 ± 1.3	0.7 ± 1.7	F(1,15)=1.1 p = 0.31	F(2,30)=16 p = 0.00	F(2,30)=0.6 p=0.54

4 Discussion

The results of this study successfully proofed the concept, that opioid induced ventilatory depression can be selectively antagonized in humans by co-administering an ampakine. While naloxone antagonized both opioid induced ventilatory depression and analgesia, CX717 selectively antagonized ventilatory depression.

This is the first successful translation of a selective antagonism of opioid-induced ventilatory depression from animals [4] to humans. It follows several unsuccessful attempts to translate similarly aimed but molecularly alternative mechanisms from animal research [22, 27] to humans [48-49]. Previously explored mechanisms targeting the serotonin HT_4, HT_{1A} or dopamine D_1 receptor systems [50], employed enhancement of respiratory rhythm at receptor sites in the pre-Bötzinger complex that are not expressed in the spinal cord or other neuronal structures relevant for mediating opioid-induced analgesia. The present study shows that there is a possibility to counter opioid effects on respiration without impeding analgesic opioid effects. Though receptor sites are also expressed outside the pre-Bötzinger complex in the central nervous system ampakines are a promising alternative, as they were not found to counteract opioid-induced analgesia [4] for reasons not yet completely understood. Additional approaches to selectively counteract opioid-induced ventilatory depression include the use of the microglial inhibitor minocycline [51], which has not yet been tried in humans, and the unspecific respiratory stimulant doxapram [52] not clinically used due to its side effect profile (hypertension, tachycardia, tremor, sweating, panic attacks, convulsions). The multitude of attempts to find a selective antidote counteracting opioid induced ventilatory depression underlines the clinical relevance of such an antidote.

Data from animal studies suggests that opioids exert their ventilatory depressive effects in part by their direct action at μ-opioid receptors expressed on neurons of the pre-Bötzinger complex, which is a small area in the ventrolateral medulla that is critical for the generation of inspiratory rhythm, in addition to other respiratory nuclei that modulate pre-Bötzinger function [53]. The neurotransmitter glutamate, acting via

AMPA receptors, is a critical component for the generation of respiratory rhythm within the pre-Bötzinger complex [31, 54-55]. Thus, mechanistically, a significant component of the CX717 effect can be explained by accentuation of AMPA receptor-mediated glutamatergic excitation that will counteract the opioid receptor-mediated suppression of pre-Bötzinger neuronal excitability [4].

Reversing effects of CX717 on alfentanil induced ventilatory depression were captured by all measured ventilatory parameters (Figure 7, Figure 8 and Table 2). The combined measurement of respiratory rate and CO_2 responsiveness to study opioid effects on ventilation is a well established approach [56-59] to provide clinically meaningful data [60]. With respect to ventilatory depression, it is thought that opioids with slower receptor binding, e.g., morphine, may be safer than those that bind more quickly, i.e., alfentanil and remifentanil, despite equianalgesic effects [61]. It is suggested that with a gradual increase in opioid levels, i.e., due to administration of a slowly equilibrating opioid such as morphine, progressive respiratory depression causes gradual hypercapnia that contributes to the maintenance of respiration [61]. In contrast, ventilatory depression induced by a rapidly equilibrating opioid, such as alfentanil, follows a typical time related sequence until complete apnea is observed [62]. It starts with a (i) reduction in respiratory rate with partial compensation of tidal volume. This is followed by (ii) a respiration that is only "kicked off" by external stimuli such as noise or pain. Subsequently (iii), the patient "forgets" respiration but can be asked to breathe [63]. Finally (iv), complete apnea occurs where in spite of external stimuli or commands the patient cannot take a deep breath.

Ampakine co-administration with opioid analgesics may be particularly beneficial in a setting requiring rapid opioid titration such as the post-surgical recovery room or ward. These settings pose an imminent risk of severe and sometimes fatal opioid-induced ventilatory depression [17]. Preventive administration of CX717 opens a possibility to significantly increase the therapeutic window of opioids and reduce the risk of severe respiratory depression. However, the present oral formulation of CX717 is not yet ideally suited for the post-surgical setting because maximum plasma concentrations peak at 2 h after administration and oral administration is often not

possible in the peri-operative setting. Therefore, the development of an intravenous formulation will be necessary to meet this clinical demand.

The examined dose of CX717, by some measures, did not completely reverse respiratory depression induced by one particular target concentration of alfentanil. Therefore, various and higher doses of the ampakine should be studied against various opioid doses. The positive outcome of this proof-of-concept is encouraging for further developing CX717 for its clinical use. In this sense it is noteworthy that a higher dose of CX717 (2100 mg) has been found tolerable (unpublished data).

Higher doses of the ampakine raise the question of interference with analgesia because analgesic effects of alfentanil were slightly reduced during the CX717 condition as compared to the placebo condition. This effect was non-significant and therefore observed at a lower statistical power of 0.45 for the effects of CX717 on the alfentanil effects on electrical pain, and of only 0.1 for heat pain. A non-significant effect indicates that the power was too low to observe it at $p < 0.05$, and increasing the power would require more observations. In the present case, to obtain the necessary power of 0.8 to observe the decrease in alfentanil analgesia at $p < 0.05$, a minimum of 129 subjects would be needed. This is a post-hoc case-number calculation based on the observed difference (CX717 versus placebo) in the percent changes from baseline of 18.7 ± 75.4% for electrical pain tolerance. With heat pain, 258 cases would be needed to find the difference between placebo and CX717 conditions of 1.8 ± 10.3% in the increase in pain tolerance statistically significant.

However, results from animal research [4] encourage the expectation that the effect size for reduction of analgesia stays favourably smaller than that for reduction of ventilatory depression. That is, the effect of CX717 on opioid induced ventilatory depression could be gradually increased with doses between 5 – 33 mg/kg. Importantly, 15 mg/kg CX717 rescued Sprague-Dawley rats from a lethal dose of 80 µg/kg fentanyl. Significantly, CX717 countered fentanyl-induced depression of respiratory frequency without suppressing analgesia [4]. This supports the expectation that the selectivity of the antagonism for respiratory but not analgesic opioid effects will persist when higher opioid and CX717 dosages will be administered.

Although heat-pain tolerance values did not return to the expected values of the baseline measurements after administration of naloxone, this is explainable by the so called re-kindling effect [64] and should not be attributed to an incompetence of the method to capture opioid antagonistic effects. The stimulated area remains sensitized by provoking the same area with heat during repetitive measurements, re-kindling even with a previous non-painful stimulus is in this situation sufficient to activate sensitized nociceptors. Nevertheless, the effect is reflected in both the verum and placebo condition in our study and could thus not be attributed to the study medication. Re-kindling in the context of the here used model to quantify heat pain tolerance in combination with capsaicin pre-application is examined at the present in a continuative study, where the effects of different time intervals and number of repetitions on heat pain tolerance assessment are being investigated.

In conclusion, we successfully demonstrated the principle of ampakine mediated potentiation of AMPA-type glutamate receptors for prevention of opioid induced respiratory effects from animal research into humans. However, the principle is not yet fully clinically applicable. There is a need for additional testing of higher doses of CX717 and opioids. On the other hand, there is need for an intravenous formulation for administration in humans in pertinent clinical settings. Nevertheless, the ampakine CX717 seems to be the first selective antidote to counteract opioid-induced ventilatory depression without decreasing analgesia in humans.

5 Abstract

Despite sensible guidelines for the use of opioid analgesics, respiratory depression remains a significant risk with a possibility of fatal outcomes. Clinicians need to find a balance of analgesia with manageable respiratory effects. The ampakine CX717 (Cortex Pharmaceuticals, Irvine, CA, USA), an allosteric enhancer of glutamate-stimulated AMPA receptor activation, has been shown to counteract opioid-induced respiratory depression in rats while preserving opioid-induced analgesia.

Adopting a translational approach, we orally administered 1500 mg of CX717 to 16 male healthy volunteers in a placebo controlled double-blind study. Starting 100 min after CX717 or placebo intake, alfentanil was administered by computerized intravenous infusion targeting a plateau of effective alfentanil plasma concentrations of 100 ng/ml. One hour after start of opioid infusion, its effects were antagonized by intravenous injection of 1.6 mg of the classical opioid antidote naloxone.

Respiration was quantified prior to drug administration (baseline), during alfentanil infusion and after naloxone administration by (i) counting the spontaneous respiratory frequency at rest and (ii) by employing hypercapnic challenge with CO_2 rebreathing that assessed the expiratory volume at a carbon dioxide concentration in the breathable air of 55% (VE_{55}). Pain was quantified at the same time points, immediately after assessment of respiratory parameters, by (i) measuring the tolerance to electrical stimuli (5 Hz sine increased by 0.2 mA/s from 0 to 20 mA and applied via two gold electrodes placed on the medial and lateral side of the mid-phalanx of the right middle finger) and (ii) by measuring the tolerance to heat (increased by 0.3°C/s from 32 to 52.5°C applied to a 3 x 3 cm^2 skin area of the left volar forearm, after sensitization with 0.15 g capsaicin cream 0.1%).

CX717 was tolerated by all subjects without side effects that would have required medical intervention. We observed that CX717 was approximately as effective as naloxone in reversing the opioid induced reduction of the respiratory frequency. Despite the presence of high plasma alfentanil concentrations, the respiratory frequency decreased only by 8.9 ± 22.4% when CX717 was pre-administered, which

was comparable to the 7.0 ± 19.3% decrease observed after administration of naloxone. In contrast, after placebo pre-administration the respiratory rate decreased by 30.0 ± 21.3% (p=0.0054 for CX717 versus placebo). In agreement with this, periods of a very low respiratory frequency of ≤ 4 min^{-1} under alfentanil alone were shortened by ampakine pre-dosing by 52.9% (p=0.0182 for CX717 versus placebo). Furthermore, VE_{55} was decreased during alfentanil infusion by 55.9 ± 16.7% under placebo pre-administration but only by 46.0 ± 18.1% under CX717 pre-administration (p=0.017 for CX717 versus placebo).

Most importantly, in contrast to naloxone, CX717 had no effect on opioid induced analgesia. Alfentanil increased the pain tolerance to electrical stimuli by 68.7 ± 59.5% with placebo pre-administration. With CX717 pre-administration, the increase of the electrical pain tolerance was similar (54.6 ± 56.7%, p=0.1 for CX717 versus placebo). Similarly, alfentanil increased the heat pain tolerance threshold by 24.6 ± 10.0% with placebo pre-administration. Ampakine co-administration had also no effect on the increase of the heat pain tolerance of the capsaicin-sensitized skin (23.1 ± 8.3%, p=0.46 for CX717 versus placebo).

The results of this study allow us to draw the conclusion, that opioid induced ventilatory depression can be selectively antagonized in humans by co-administering an ampakine. This is the first successful translation of a selective antagonism of opioid-induced respiratory depression from animal research into application in humans.

Ampakines, namely CX717, thus are the first selective antidote for opioid-induced respiratory depression without loss of analgesia, available for the use in humans.

6 Deutsche Zusammenfassung

Die Atemdepression ist die gefährlichste Nebenwirkung von Opioidanalgetika. Ihre selektive Antagonisierung, ohne die analgetischen Wirkungen zu beeinflussen, wäre ein bedeutender therapeutischer Fortschritt. Molekularbiologische und tierexperimentelle Befunden zeigten, dass möglicherweise das Ampakin CX717 (Cortex Pharmaceuticals, Irvine, CA, USA) dafür geeignet ist. Es verstärkte über eine allosterische Bindungsstelle die Wirkung von Glutamat am AMPA-Rezeptor und konnte in einem Tiermodell an Ratten die opioid-induzierte Atemdepression bei gleichbleibender Analgesie verhindern.

In einem translationalen Ansatz führten wir eine doppelblind angelegte, placebokontrollierte Studie in 16 gesunden männlichen Probanden durch, denen 1500 mg CX717 oral verabreicht wurden, und 100 min später computergesteuert Alfentanil über 2 h infundiert wurde (Alfentanil-Plasmazielkonzentration im „steady-state“ von 100 ng/ml). Eine Stunde nach Start der Opioid-Infusion wurden die Wirkungen des Alfentanils durch die intravenöse Gabe von 1.6 mg Naloxon antagonisiert. Die Wirkungen des Opioids auf Atmung und Schmerz wurden jeweils mittels zweier experimenteller Parameter quantifiziert. Die Atemdepression wurde mittels Aufzeichnung der spontanen Atemfrequenz in Ruhe sowie mittels CO_2-Rückatemversuch gemessen, bei welcher das exspiratorische Volumen bei einer CO_2-Konzentration von 55% in der Einatemluft (VE_{55}) als Hauptzielparameter diente. Die Schmerzwahrnehmung wurde als Schmerztoleranz auf elektrische Stimuli (sinusförmige Reize einer Stromstärke von 0-20 mA; Applikation über zwei Goldelektroden am mittleren Fingerendgelenks des rechten Mittelfingers) sowie als Toleranz auf Hitze-induzierten Schmerz gemessen (Temperaturanstieg: 0.3°C/s von 32.5 -52.5, Applikation über eine 3 x 3 cm² große Hautfläche an der volaren Seite des linken Unterarms, nach Sensibilisierung der Haut mit 0.15 g Capsaicin Creme 0.1%).

Während der Alfentanil-Infusion (Plasmakonzentrationen ca. XXX) verminderte sich die Atemfrequenz unter Gabe von CX717 nur um 8.9 ± 22.4%, vergleichbar mit 7.0 ± 19.3% Rückgang nach Gabe von Naloxon. Dagegen kam es unter Placebo zu einer Senkung

der Atemfrequenz um 30 ± 21.3% (p=0.0054 für CX717 versus Placebo). Zusätzlich wurden Perioden mit sehr niedriger Atemfrequenz von ≤ 4 min^{-1} unter Alfentanil nach pre-Medikation mit CX717 um 52.9% verkürzt (p=0.0182 für CX717 versus Placebo). Darüber hinaus war die VE_{55} während der Alfentanil Infusion nach Placebo-Gabe um 55.9 ± 16.7% verringert, während es nach Gabe von CX717 nur zu einer Verminderung von 46.0 ± 18.1% kam (p=0.017 für CX717 versus Placebo). Im Gegensatz zu der signifikanten Antagonisierung der atemdepressiven Wirkungen von Alfentanil hatte CX717 keine statistisch signifikanten Auswirkungen auf die alfentanilinduzierte Analgesie, während Naloxon unselektiv auch die Analgesie antagonisierte. Alfentanil allein erhöhte die Schmerztoleranz auf elektrische Stimuli um 68.7 ± 59.5%. Unter Gabe von CX717 war die Steigerung der elektrischen Schmerztoleranz ähnlich (54.6 ± 56.7%, p=0.1 für CX717 versus Placebo). Entsprechend erhöhte die alleinige Gabe von Alfentanil die Hitze-Toleranz-Schwelle um 24.6 ± 10.0%, während eine pre-Medikation mit CX717 auch hier keinen Einfluss auf den Anstieg der Hitzetoleranz hatte (23.1 ± 8.3%, p=0.46 für CX717 versus Placebo).

Aus diesen Ergebnissen kann man einen selektiven Antagonismus der atemdepressiven Wirkungen des Opioids Alfentanil durch das Ampakin CX717 ableiten. Die vorliegende Arbeit stellt also eine erfolgreiche Translation tierexperimenteller Forschungsergebnisse in den Menschen dar. CX717 stellt somit das erste selektive Antidot für die Opioid-induzierte Atemdepression beim Menschen dar, das nicht zu einem Verlust der Analgesie führt.

7 Literature

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8 Appendix

8.1 Abbreviations

e.g.	Example given
F	F-value
i.e.	Id est
kg	Kilogram
kPa	Kilopascal
l	Liter
LC-MS/MS	Liquid chromatographic tandem mass spectrometric
m	Meter
mA	Milliampere
mg	Milligram
ml	Milliliter
min	Minute
mM	Millimolar
mmHG	Millimeters of Mercury
pCO_2	CO_2 partial pressure
s	Second
$t_{½}(k_{e0})$	Equilibration time
µg	Microgram
VAS	Visual analog scale
V_E	Expiratory volume

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8.3 Publication

Oertel B.G., **Felden L.**, Tran P. V., Bradshaw M. H., Angst M. S., Schmidt H., Johnson S., Greer J. J., Geisslinger G., Varney M. A., Lötsch J. *Selective antagonism of opioid-induced ventilatory depression by an ampakine molecule in humans without loss of opioid analgesia.* Clin Pharmacol Ther, 2010; 87(2): 204-11

Walter C. **Felden L.**, and Lötsch J. *Bioequivalence criteria for transdermal fentanyl generics: do these need a relook?* Clin Pharmacokinet, 2009; 48(10): 625-33

Felden L., Walter C., Harder S., Treede R.-D., Kayser H., Drover D., Geisslinger G., and Lötsch J. *Does hydromorphone possess clinical advantages over the WHO standard morphine in personalized therapy of pain patients?* (submitted)

8.4 Ehrenwörtliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Humanmedizin zur Promotionsprüfung eingereichte Arbeit mit dem Titel „Selective antagonism of opioid-induced ventilatory depression by an ampakine molecule in humans without loss of opioid analgesia“ unter Betreuung von Herrn Prof. Dr. med. Lötsch am Zentrum der Pharmakologie, Institut für klinische Pharmakologie (Direktor: Herr Prof. Dr. med. Dr. rer. nat. Geisslinger) ohne sonstige Hilfe durchgeführt und bei der Abfassung der Dissertation keine anderen als die in der Dissertation angeführten Hilfsmittel benutzt habe. Darüber hinaus versichere ich, nicht die Hilfe einer kommerziellen Promotionsvermittlung in Anspruch genommen zu haben.

Ich habe bisher an keinem in- und ausländischen Medizinischen Fachbereich ein Gesuch um Zulassung zur Promotion eingereicht. Die vorliegende Arbeit wurde bisher nicht als Dissertation eingereicht.

Vorliegende Ergebnisse der Arbeit wurden in folgendem Publikationsorgan veröffentlicht:

Oertel B.G., Felden L., Tran P. V., Bradshaw M. H., Angst M. S., Schmidt H., Johnson S., Greer J. J., Geisslinger G., Varney M. A., Lötsch J. *Selective antagonism of opioid-induced ventilatory depression by an ampakine molecule in humans without loss of opioid analgesia.* Clin Pharmacol Ther, 2010. **87**(2): p. 204-11

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