Abstract: It is well known that in clinical practice opioids may cause skeletal muscle rigidity. Involvement of the respiratory musculature, laryngeal structures, or the chest wall may impair ventilation, resulting in hypercarbia and hypoxemia. Since muscle rigidity is clinically undesirable, the ability to synthesize the compound with less side effects could lead to the development of a new clinically valuable opioid drug. This study aimed at evaluating the antinociception, skeletal muscle (trunk) rigidity and catalepsy (muscular rigidity and immobility) of fentanyl analogs: (±)cis-3-carbomethoxy fentanyl (C) and (±)trans-3-carbomethoxy fentanyl (T) in rats. The effects were dose-dependent and abolished by pretreatment with naloxone, nonselective antagonist of opioid receptors, indicating that they are mediated via opioid receptors. C and T are less potent (2.5-3.0 and 7.9-11.1 times, respectively) than fentanyl (F) in producing antinociception, trunk rigidity and catalepsy. Further, F, C and T exhibited similar relative potencies in producing all tested effects, indicating that similar receptors are
involved in producing antinociceptive and adverse effects, most probably of µ type. No significant differences between therapeutic indices for F, C and T were found, indicating that these compounds are equally safe and tolerable in respect to the skeletal muscle rigidity. Trunk rigidity and catalepsy testing presented in this paper may be useful in studying the structure-activity relationship of opioid congeners.

Introduction

Opioids are the most common postoperative analgesic prescribed, and they have a notable adverse effect profile including respiratory depression via upper airway dilator (genioglossus and tensor palatine) (1) and respiratory pump muscles (diaphragm, intercostals) (2-3) dysfunction.

In animal models, opioids impair upper airway muscle function in a dose-dependent fashion. This effect has been shown through reduced genioglossus activity in rats (1), decreased vagal motor neuron activity in laryngeal abductors, and increased vagal motor neuron activity in laryngeal adductors (4). These opioid-induced changes result in increased upper airway resistance and possibly vocal cord closure and pharyngeal airflow obstruction (4-5). The effect of opioids on muscle function extends to those of the respiratory pump including muscles of the thorax and the diaphragm. Chest wall rigidity following opioid administration was first described in 1953 (6) and while usually associated with rapid injection, multiple agents, and large doses, it has also occurred when opioids were administered in a conservative fashion. Opioid analgesia has also been shown to increase abdominal muscle activity, (7) and this persistent expiratory muscle activity produces a rapid decrease in end-expiratory lung volume and functional residual capacity, contributing to a higher degree of atelectasis (8). This opioid-induced expiratory muscle recruitment appears not to be related to airway obstruction (9). Even the diaphragm is affected. In spontaneously ventilating rats, high-dose opioids led to diaphragm dysfunction and a reduction in phasic activity, leading to reduced tidal volume and minute ventilation; (2) similar effects have been seen in felines (4).

Fentanyl, a 4-anilidopiperidine derivative, is a potent opioid with rapid onset and short duration of action. An analgesic agent with these features is suitable for the production of conscious-sedation, neuroleptanalgesia, neuroleptanesthesia, and anesthesia. A side effect, namely rigidity of the chest, jaw, and abdominal muscles, has been noted with fentanyl and fentanyl-related compounds (10-12). Like most of the currently available strong opioid analgesics, fentanyl exerts analgesic and adverse effects primarily through the opioid µ receptors (13).
The most common approach in searching for novel drugs is structural modification of the well known compounds (14-23). Among the important properties of the opioids that can be altered by structural modification are their affinities for various types of opioid receptors, activities as agonists versus antagonists, lipid solubilities, and their susceptibility/resistance to metabolic breakdown (25-27). Since muscle rigidity is clinically undesirable, the ability to synthesize the compound with less side effects could lead to the development of a new clinically valuable opioid drug.

In our earlier work, 3-carbomethoxy fentanyl (Fig. 1) was prepared and tested for analgesic activity, body temperature response and some neurotoxic effects (loss of righting reflex, cornel reflex, etc) in rats (14-15; 20-24). It was found that (±)cis 3-carbomethoxy fentanyl and (±)trans 3-carbomethoxy fentanyl were about 2 and 10 times less potent analgesics than fentanyl, respectively. Also, both of these compounds possessed more rapid onset, as well as shorter duration of antinociception in comparison with fentanyl (21; 24). Although the efficacy, as well as structure-activity relationship (SAR) of (±)cis and (±)trans 3-carbomethoxy fentanyl with regard to antinociception and some neurotoxic effects have been previously determined, their safety in regard to skeletal muscles rigidity remained unexplored. This study is aimed at evaluating the relative safety of (±)cis and (±)trans 3-carbomethoxy fentanyl and fentanyl, by using tests for assessing skeletal muscle (trunk) rigidity and catalepsy (muscular rigidity and immobility) in rats (14, 28-32).

\[
\begin{align*}
\text{Fentanyl} & \quad (\pm)\text{cis 3-Carbomethoxy fentanyl} & \quad (\pm)\text{trans 3-Carbomethoxy fentanyl}
\end{align*}
\]

Figure 1. Fentanyl, (±)cis 3-carbomethoxy fentanyl and (±)trans 3-carbomethoxy fentanyl.
Material and methods

Animals

Wistar rats (200–250 g) of both sexes obtained from Military Farm (Belgrade, Serbia) were used. All experiments were approved by the Ethics Committee for Animal Research And Welfare of Faculty of Medicine, University of Belgrade (permission. N° 5057/2). All experiments were approved by the Ethical Council for Protection of Experimental Animals of the Ministry of Agriculture, Forestry and Water Management of the Republic of Serbia, which operates in accordance with Animal Welfare Law of our country and National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978) for the use of animals in research. The animals were housed in groups of 4 in plexiglas cages (42.5 × 27 × 19 cm) under standard conditions: temperature of 22°C ± 1°C, and a 12/12 h light/dark cycle with lights on at 08.00 h. Food pallets and tap water were available ad libitum, except during the experimental procedure. Prior to each experiment the animals were habituated to the handling and experimental procedures for at least three consecutive days. Experiments were done in a sound–proofed, diffusely illuminated room maintained at a temperature of 22±1°C. They were performed at the same time of the day between 9:00 and 13:00 h to avoid diurnal variation in behavioral tests. The animals were unrestrained during all experimental procedures, except antinociception testing. Experimental groups consisted of 6–8 rats. Each animal was used only once and was killed with i.p. injection of sodium thiopental (33-34).

Experimental testing

Antinociceptive activity was determined by tail-immersion test (35). The distal 5 cm of the tail was immersed in a warm water bath (55±0.5°C) and the time for tail-withdrawal was measured as a response latency. In order to minimize tissue damage by repeated testing, a cut-off time of 6 s was adopted. An antinociceptive effect was said to have occurred if postdrug response latency was ≥ 6s. Catalepsy was defined as the failure of the animal to move within 60 s from a position in which the forepaws and hind paws were placed on bars 10 cm from the floor (32, 36). Trunk rigidity was assessed by palpation (31). Testing was performed once before and at 5, 20, 40, 60, 90 etc. min after i.p. drug (or the vehicle in the control group) injection by a two observers unaware of the pharmacological treatment. The data are expressed quantally as the number of animals
in which the antinociception was observed versus total number of animals receiving the same treatment. Experimental groups consisted of 6–8 rats.

**Drugs administration**

Fentanyl citrate (ICN Yugoslavia, Belgrade, Yugoslavia) and (±)cis and (±)trans 3-carbomethoxy fentanyl oxalate were dissolved in saline and injected i.p. in a final volume of 2 ml/kg. Both (±)cis and (±)trans 3-carbomethoxy fentanyl were examined as a racemic mixture. Doses of the drugs were calculated for the free base. Naloxone hydrochloride (Sigma Chemical Co. St. Louis, USA) was also dissolved in saline, and injected s.c. (1 mg/kg) in the back before the i.p. injection of the test compound in the same volume. In order to test whether saline injection has any effect on nociception or toxic behavior, 2 ml/kg of saline were administrated i.p. in a control group of rats.

**Statistical analysis**

To permit direct comparison of different compounds and different effects, basic data for each animal were transformed to a quantal response (presence or absence of expected drug effect). For each effect and each dose maximum response obtained during time of measurement was used for evaluation. Then, computations were done according to the methods of Tallarida and Murray (37). First, the percents of animals responding were converted to probits and plotted against the log dose. Then, the Litchfield & Wilcoxon procedure was used to calculate ED$_{50}$catalepsy, ED$_{50}$trunk rigidity (median doses that induce catalepsy/trunk rigidity) and ED$_{50}$antinociception (median antinociceptive dose) values from corresponding quantal dose–response curves. Additionally, when data for a second quantal dose–response curve was entered, the same procedure calculated the potency ratio [with confidence limits (CL)] for corresponding curves. In that way, relative potencies for fentanyl, (±)cis 3-carbomethoxy fentanyl and (±)trans 3-carbomethoxy fentanyl were calculated for antinociceptive, as well as toxic effects. Relative potency estimates were considered statistically significantly different when 95% CL did not overlap 1.0. Also, the potency ratios between ED$_{50}$catalepsy/ED$_{50}$antinociception and ED$_{50}$trunk rigidity /ED$_{50}$antinociception, denoted as the TI (therapeutic index), for each drug were determined. If the 95% confidence interval for a TI fails to include 1.0, then twoTD$_{50}$s are significantly different.
Results

Fentanyl (F; 0.007-0.120 mg/kg; i.p.), (±)cis 3-carbomethoxy fentanyl (C; 0.016-0.326 mg/kg; i.p.) and (±)trans 3-carbomethoxy fentanyl (T; 0.08-1.22 mg/kg; i.p) produced dose-dependent increase in antinociception, catalepsy and trunk rigidity (Fig. 2). The median effective doses (ED\textsubscript{50}s) for antinociception, catalepsy and trunk rigidity, and the relative potencies for F, C and T are presented in Table 1. The ED\textsubscript{50}s for C and T are significantly higher (p<0.05) in comparison with the corresponding values for F, indicating that C and T are less potent than F in producing both antinociception

Figure 2. Log dose-probit curves for antinociception (tail withdrawal test), catalepsy and trunk rigidity for fentanyl (A), (±)cis 3-carbomethoxy fentanyl (B) and (±)trans 3-carbomethoxy fentanyl (C) in rats. For each effect and each dose, maximum response obtained during time of measurement was transformed to a probit value. Each point represents the probit value obtained from 6-8 rats.
Table 1. Median effective doses (ED$_{50}$), relative potencies and therapeutic indices with 95% confidence limits (95% CL) for fentanyl, (±)cis 3-carbomethoxy fentanyl and (±)trans 3-carbomethoxy fentanyl in inducing antinociception, catalepsy and trunk rigidity in rats.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Fentanyl (0.0073-0.12 mg/kg)</th>
<th>(±)cis 3-Carbomethoxy fentanyl (0.016-0.33 mg/kg)</th>
<th>(±)trans 3-Carbomethoxy fentanyl (0.08-1.22 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ED$_{50}$ (95% confidence interval)</td>
<td>Relative potency (95% CL)$^1$</td>
<td>TI (95% CL)$^1$</td>
</tr>
<tr>
<td>Antinociception$^2$</td>
<td>0.01 (0.007-0.015)</td>
<td>1 (0.017-0.035)</td>
<td>0.49$^*$ (0.24-0.67)</td>
</tr>
<tr>
<td>Catalepsy$^3$</td>
<td>0.067 (0.06-0.08)</td>
<td>1 (4.5-10.1)</td>
<td>0.21 (0.14-0.29)</td>
</tr>
<tr>
<td>Trunk rigidity</td>
<td>0.07 (0.05-0.10)</td>
<td>1 (4.3-12.0)</td>
<td>0.19 (0.13-0.28)</td>
</tr>
</tbody>
</table>

ED$_{50}$ = median effective dose.
Median effective dose for each effect and each compound was calculated by using 3 doses. One dose is tested in at least 6 rats (Litchfield & Wilcoxon I test).

$^1$ Therapeutic index (TI) is calculated as ED$_{50}$catalepsy/muscle rigidity/ED$_{50}$antinociception potency ratio for each drug.

$^2$ criterion ≥6 s.

$^3$ criterion ≥60 s.

$^*$ P < 0.05, Litchfield & Wilcoxon II test. Relative potency estimates were considered statistically significant when 95% CL did not overlap 1.0.

$^{*}$ P < 0.05, Litchfield & Wilcoxon II test. If 95% CL for a TI fails to include 1.0, then ED$_{50}$catalepsy/muscle rigidity and ED$_{50}$antinociception are statistically different.
and effects on skeletal muscles. Also, T was significantly less potent (p<0.05) in producing all observed effects than C. The potency ratios indicate that C and T are less potent analgesics (2.5 and 7.9 times, respectively), in comparison with F. C and T are 3.0 and 11.1 times less potent than F in producing catalepsy, respectively. Also, C and T are 2.7 and 9.1 times less potent than F in producing trunk rigidity. T is 3.2, 3.4 and 3.3 times less potent than C in inducing antinociception, catalepsy and trunk rigidity, respectively (not shown).

Each of tested compounds, exhibited similar (p>0.05) relative potencies in producing all effects tested (95% confidence intervals overlap) (Table 1).

The ED<sub>50</sub><sub>antinocicception</sub> values for F, C, and T are significantly lower (p<0.05) than their ED<sub>50</sub><sub>catalepsy</sub> or ED<sub>50</sub><sub>trunk rigidity</sub> values (Table 1). The greater the TI value is, the greater is observed drug tolerability. Within each effect, there are no significant differences (p>0.05) between TIs for F, C and T. That means that these compound are equally tolerable in regard to observed effects on catalepsy and trunk rigidity and the difference between them is in potency only.

Naloxone hydrochloride (1 mg/kg; s.c.) given 10 min before i.p. injection of 8xED<sub>50</sub> of F, C and T abolished all the effects tested (not shown).

In control experiments, i.p. injection of saline (0.2 ml/kg) had no effect on the animal's behavior, as well as tail immersion latency (P>0.05); the latencies before and after saline injection were found to be 2.40 ± 0.30 and 2.51 ± 0.33 s, respectively (n = 8) (not shown).

**Discussion**

Opioid drugs, such as morphine, are a mainstay for pain relief for patients around the world. They are used in a wide variety of clinical situations, for example, after surgery and in control of pain due to cancer. Respiratory depression limits the use of opioid analgesia. Chest wall rigidity is a serious complication that must be diagnosed and treated immediately. Respiratory and cardiovascular complications may develop from hypoxemia and hypercarbia with resulting morbidity and mortality.

The present experiments are an extension of our previous study on the antinociceptive and toxic effects of the newly synthesized fentanyl (F) analogues, (±)cis 3-carbomethoxy fentanyl (C) and (±)trans 3-carbomethoxy fentanyl (T) (15, 20, 22, 24), and deal with their effect on trunk rigidity and catalepsy in rats. Examination of the relationship between different opioid effects may lead to a better understanding of the mechanism and sites of action of opioids. Our previous studies has also compared the dose-effect
relationships for morphine and fentanyl analogues between antinociception and effect on body temperature (19). However, neither muscle rigidity nor lack of spontaneous movement plus rigidity (catalepsy) has been fully examined or directly compared with antinociception.

The obtained results confirmed previous finding that C and T are less potent analgesics (about 2.5 and 7.9 times, respectively), in comparison with F. All three compounds tested produced dose-dependent increase in antinociception, catalepsy and trunk rigidity, with the similar median effective doses for catalepsy and trunk rigidity. Both, antinociceptive and effects on skeletal muscle were abolished by pretreatment with naloxone, nonselective antagonist of opioid receptors, indicating that they are mediated by opioid receptors. It was revealed that F, C and T exhibited similar relative potencies in producing all effects tested. If a series of related agonists exhibits identical relative potencies in producing distinct effects, it is likely that these effects are mediated by similar or identical receptor molecules (13). In the case of F, C and T, they are of \( \mu \) type, most probably. Most of the currently available opioid analgesics exert their analgesic and adverse effects primarily through the opioid \( \mu \) receptors. However, individual strong opioids may interact, at least in part, with different opioid receptor subpopulations or modulate \( \mu \) opioid receptor signaling in different ways (38-40), that may improve tolerability (26; 41). Consistent with our finding, Vankova (42) demonstrated that in rats opioid-induced muscle rigidity is primarily due to the activation of central \( \mu \) receptors, while supraspinal \( \delta_1 \) and \( \kappa_1 \) opioid receptors may attenuate this effect. Hajiha et al. (1) suggested that in rats \( \mu \)-opioid receptor stimulation suppresses motor output from a central respiratory motoneuronal pool that activates genioglossus muscle, and this may underlie the clinical concern regarding adverse upper airway function with \( \mu \)-opioid analgesics. The role of \( \mu \) opioid receptors in catalepsy has been previously reported (43). Intra-accumbens infusion of PLO17, a selective \( \mu \)-opioid receptor agonist, elicited motor inhibition with rigidity and catalepsy, while infusion of (D-Ala\(_2\))deltorphin II, a selective \( \delta \)-opioid receptor agonist evoked dose-dependent motor stimulation characterized by locomotion, sniffing, and oral stereotypies.

There are no significant differences between ED\(_{50}\)catalepsy and ED\(_{50}\)trunk rigidity values for all three compound tested. The ED\(_{50}\)antinociception values for F, C, and T are significantly (6.8 to 9.0 times) lower than their ED\(_{50}\)catalepsy/trunk rigidity values. There are no significant differences between therapeutic indices for F, C and T, which means that these compounds are equally tolerable in regard to the observed effects on skeletal muscle, and the difference between them is in the potency.
In conclusion, F, C, and T are equally tolerable drugs in respect to the effects on catalepsy and trunk rigidity. Also, F, C and T exhibited similar relative potencies in producing all effects evaluated, and the structure-activity relationship on effects on skeletal muscle of fentanyl analogs obtained by introducing carbomethoxy group in the position 3 of the piperidine ring, parallels the structure-activity relationship on analgesia. All of these taken together, might suggest that similar receptors are involved in producing both antinociceptive and effects on skeletal muscle of F, C and T, most probably of μ type.

Animal testing presented in this paper consists of procedures which can be performed easily and in parallel manner providing several useful pharmacological informations regarding safety and tolerability, and also, could be indicative whether the observed drug effects are mediated by similar or different receptors. Therefore, we recommend it as a useful approach in studying the structure-activity relationship of opioid congeners.

Acknowledgments

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References