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Antagonistic targeting of the histamine H_3 receptor decreases caloric intake in higher mammalian species

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ABSTRACT

The main purpose of this study was to examine the effects of a selective histamine H_3 receptor antagonist, NNC 38-1202, on caloric intake in pigs and in rhesus monkeys. The compound was given intragastrically (5 or 15 mg/kg), to normal pigs ($n = 7$) and subcutaneously (1 or 0.1 mg/kg) to obese rhesus monkeys ($n = 9$). The energy intake recorded following administration of vehicle to the same animals served as control for the effect of the compound. In addition, rhesus monkey and pig histamine H_3 receptors were cloned from hypothalamic tissues and expressed in mammalian cell lines. The in vitro antagonist potencies of NNC 38-1202 at the H_3 receptors were determined using a functional GTPγS binding assay.

Porcine and human H_3 receptors were found to have 93.3% identity at the amino acid level and the close homology between the monkey and human H_3 receptors (98.4% identity) was confirmed. The antagonist potencies of NNC 38-1202 at the porcine, monkey and human histamine H_3 receptors were high as evidenced by K_i-values being clearly below 20 nM, whereas the K_i-value on the rat H_3 receptor was significantly higher (56 ± 6.0 nM). NNC 38-1202, given to pigs in a dose of 15 mg/kg, produced a significant ($p < 0.05$) reduction (55%) of calorie intake compared with vehicle alone, (132.6 ± 10.0 kcal/kg day versus 59.7 ± 10.2 kcal/kg day). In rhesus monkeys administration of 0.1 and 1 mg/kg decreased ($p < 0.05$) average calorie intakes by 40 and 75%, respectively.

In conclusion, the present study demonstrates that antagonistic targeting of the histamine H_3 receptor decreases caloric intake in higher mammalian species.

1. Introduction

Neuronal histamine plays a central role in the regulation of food intake and body weight homeostasis [1,2]. This effect is thought to be mediated by signaling from central histamine H_1 receptors [3] located post-synaptically in the complex hypothalamic network of neurons that control calorie intake and thermogenesis [4]. However, due to the widespread

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Abbreviations: i.g., intra gastric; PCR, polymerase chain reaction; HEK-cells, human embryonic kidney-cells

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distribution of the histamine H_1 receptor outside the brain, direct targeting of this receptor with a histamine H_1 receptor agonist would result in a number of unspecific and undesirable effects. Instead, selective targeting of the histamine H_3 receptor represents a more promising approach, since the expression of this pre-synaptic receptor is essentially confined to the central nervous system [5]. Here, one of its main functions is to exert a negative feed-back regulation of intra-synaptic histamine levels [6]. Thus, by inhibiting the actions of the H_3 receptor with a selective H_3 receptor antagonist, the intra-synaptic histamine level would increase and result in an increased signaling from histamine H_1 receptors and an inhibition of food intake.

It has previously been demonstrated that administration of a selective histamine H_3 receptor antagonist to rodents is associated with an increase in paraventricular histamine levels [7,8], suppression of food intake and a decreased body weight [7–9].

The main purpose of this study was to investigate whether the effects observed in rodents would also be found in higher mammalian species, as indicated by a decrease in caloric intake following administration of a selective histamine H_3 receptor antagonist. NNC 0038-0000-1202, abbreviated NNC 38-1202, is a cinnamic amide, (*E*)-1-((*S*)-2-((pyrrolidin-1-yl)methyl)pyrrolidin-1-yl)-3-(4-(trifluoromethyl)phenyl)propenone [10] and has a plasma half-life in rats of 226 min following i.v. administration. The oral availability of the compound is close to 100% in rodents and it also achieves good penetration of the hypothalamic tissue.

Here we show that NNC 38-1202 is a potent histamine H_3 antagonist at pig and monkey H_3 receptors, and that it decreases caloric intake in both species.

2. Methods

2.1. Cloning and expression of porcine and rhesus monkey histamine H_3 receptors

Porcine and rhesus monkey histamine H_3 receptors were cloned using polymerase chain reaction (PCR) based on homology with the previously reported human [5,11] and rat [11,12] sequences. At the time when these experiments were initiated, results from the first cloning of the monkey histamine receptor [13] were not in the public domain. PCRs were performed on cDNA generated from hypothalamic porcine and rhesus monkey tissue.

The cDNAs of the monkey and pig H_3 receptors were inserted into the mammalian cell expression vector pcDNA3.1 (Invitrogen, Carslbad, CA, USA), transfected into HEK-293 cells. Stable HEK-293 cell lines expressing either the porcine or the monkey histamine H_3 receptor were established, essentially as described previously for the human and rat histamine H_3 receptors [11].

2.2. The in vitro antagonist potency of NNC 38-1202

The in vitro antagonist potency of NNC 38-1202 at various species variants of the histamine H_3 receptor was studied using a functional [^{35}S]GTPγ[S] binding assay from which K_i values were generated as an estimate of antagonism, essentially as described previously [10]. In the same article [10] it is also shown that NNC 38-1202 almost exclusively binds to the H_3 receptor, whereas binding to other histamine receptors is negligible.

2.3. Animals

2.3.1. Pigs

On arrival, seven Danish Landrace pigs (Gundsøgaard, Roskilde, Denmark) with a mean body weight of about 35 kg, previously accustomed to two meals a day, were first trained to have free access to a standard pig diet for daily periods of 5 h. The diet (Mini-Pig, SDS, Witham, Essex, UK) had a calculated gross energy content of 3.3 Mcal/kg (13.8 MJ/kg). After about 2 weeks of adaptation to the experimental facilities, and an overnight fast, pigs were fitted with intra-gastric (i.g.), and in some animals also vascular catheters, as previously described [14].

Following surgery, a 7-day recovery period was allowed before initiation of experiments. Each pig underwent three experiment-sessions with i.g. administration of vehicle or NNC 38-1202, at doses of either 5 or 15 mg/kg, shortly before presentation of food at 07:00 h. The order in which treatments were given was randomized so that all three treatments were represented in all three sessions. These three experiment-sessions were separated by periods of three days in which pigs did not receive any compound. Food intake was recorded once every hour between 07:00 and 12:00 h. Spillage was prevented by presenting the food in relative deep trough.

2.3.2. Rhesus monkeys

Nine obese rhesus monkeys (*Macaca mulatta*), five females and four males, with a mean body weight of 9.2 ± 0.8 kg were recruited from Dr. Hansen's monkey colony (University of South Florida, Tampa, USA). Each monkey underwent seven experiment-sessions, within a total time period of 5 weeks, in which subcutaneous injections of vehicle or NNC 38-1202, at dose levels of either 0.1 or 1 mg/kg, were given shortly before presentation of food at 08.30 h. Food was provided in the form of biscuits (monkey chow, Purina Mills Inc. St. Louis, MO, USA) containing 25 kcal/biscuit and was freely available between 08:30 and 16:30 h. The monkeys were well acquainted with this feeding-regime and diet. The number of biscuits consumed during this period of time was recorded once every two hours. The final cumulative 8 h food intake was adjusted for eventual spillage.

Vehicle was administered in three of the seven sessions. NNC 38-1202, in a dose of 0.1 mg/kg, was also given on three occasions, whereas 1 mg/kg was only injected once in each animal. This was due to the strong inhibition of food intake achieved with this dose, causing complete anorexia in two individual monkeys.

In the cases where the same treatment was repeated in the same animal a mean response for this animal and time of observation was calculated and was treated as one independent observation in the further statistical analyses of data.

2.3.3. Animal ethics

All animal experiments were concordant with the Declaration of Helsinki and were approved by the local ethical committies.

2.3.4. *Unspecific effects*

In association with administration of NNC 38-1202 the reaction of each individual animal was followed carefully and technical staff was instructed to note eventual deviations from normal behavior.

2.4. *Calculations and statistics*

The sequence alignment and the phylogenic relation between the histamine H_3 receptor in humans, rhesus monkeys, pigs and rats were calculated using the VectorNTI® software (Invitrogen, Carlsbad, CA, USA). The homology and identity calculations were performed using the SIM program (http://www.expasy.org/).

The caloric intake was assessed by multiplying the amount of food eaten by its estimated content of gross energy and was expressed per kg body weight. Statistical tests for potential differences between treatments were performed at the final time point of the experimental sessions. GraphPad Prism version 4.0 (GraphPad® Software, San Diego, CA, USA) or SAS version 9.1 (SAS Institute, Cary, NC, USA) software were used for statistical tests and graphical presentation of data. Potential differences between treatment groups were tested by a two-way analyses of variance followed by Dunnett's post hoc test for multiple comparisons. In this model the effect of treatment and individual animal were regarded as prime sources of variation. Comparisons having a p-value of less than 0.05 were considered to be statistically significant. Data are presented as means ± S.E.M.

3. Results

3.1. *Inter-species histamine H_3 receptor homologies*

When the amino acid sequence of the porcine histamine H_3 receptor was aligned with the previously known sequences for the human [5] monkey [13] and rat [12] variants it became evident that this receptor exhibited a high degree of homology with the other variants (Fig. 1). The amino acid sequences of

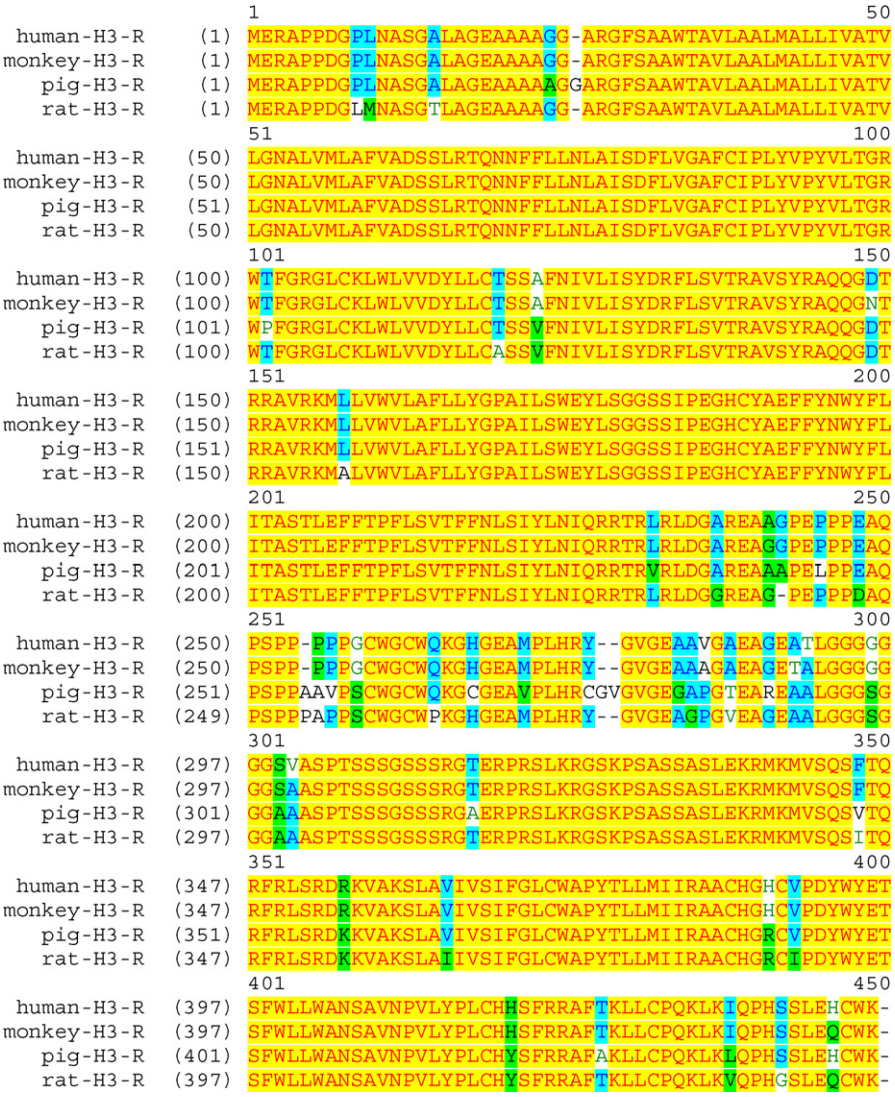

Fig. 1 – The amino acid sequence of the porcine histamine H_3 receptor together with the previously known sequences for the human, monkey and rat variants of the receptor.

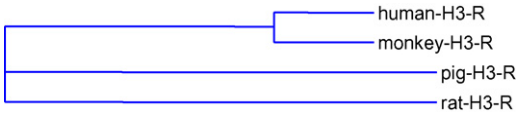

Fig. 2 – The phylogenetic relationship between histamine H_3 receptors in pigs, monkeys, rats and humans.

the porcine and human receptors share a 93.3% identity. Although this number is high it should be noted that the monkey and human H_3 receptors show an even higher degree identity (98.4%). The phylogenetic relationship between the actual receptors is depicted in (Fig. 2). Obviously, human and monkeys have had a comparatively long period of common evolution of the histamine H_3 receptor.

As reviewed by Hancock et al. [15] the amino acids 119 and 122, being situated in the binding pocket of the histamine H_3 receptor, are critical for the receptor's pharmacological properties. In the porcine H_3 receptor the amino acids in these positions were found to be T–V (threonine–valine). As can be seen from Fig. 1, the corresponding amino acids in the human and monkey H_3 receptors are T–A (threonine–alanine) whereas the rat has the combination A–V (alanine–valine).

3.2. *The in vitro antagonist potency of NNC 38-1202*

As shown in Table 1, the antagonist potency of NNC 38-1202 at monkey and human histamine H_3 receptors was high, whereas the compound was a somewhat less potent at the porcine receptor and the least potent at the rat receptor.

3.3. *Effects on caloric intake in pigs*

In pigs, administration of NNC 38-1202 in a dose of 15 mg/kg (i.g.), produced a significant ($p < 0.05$) decrease in the daily intake of energy compared with that following administration of vehicle alone (132.6 ± 10.0 kcal/kg day versus 59.7 ± 10.2 kcal/kg/day). This was equivalent to an average reduction of caloric intake of 55%. A dose of 5 mg/kg seemed also to reduce calorie intake up to 3 h but the accumulated amount consumed after 5 h (121.1 ± 9.1 kcal/kg day) was not significantly different from that associated with administration of vehicle (Fig. 3). The difference in efficacy between the two doses was clearly mirrored in the plasma exposure profiles (Fig. 4)

3.4. *Effects on caloric intake in rhesus monkeys*

When injected subcutaneously with 1 mg/kg of NNC 38-1202, monkeys responded with a significant ($p < 0.05$) reduction of

Table 1 – Antagonist potency (K_i) of NNC 0038-0000-1202 at the recombinantly expressed human, rat, pig and rhesus monkey histamine H_3 receptor

Species	n	K_i (nM)	S.E.M.
Human	39	6.7	0.50
Rhesus monkey	4	6.3	0.75
Pig	3	18	0.67
Rat	9	56	6.0

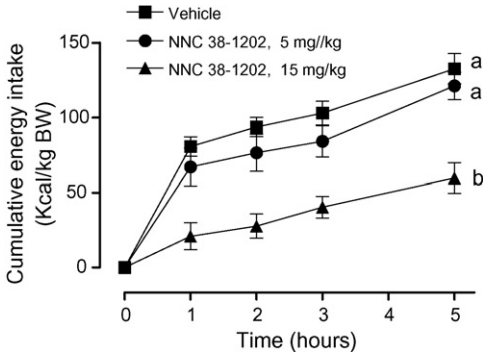

Fig. 3 – Effects of the histamine H_3 receptor antagonist NNC 38-1202 on cumulative caloric intake in pigs following single intra-gastric doses of 5 and 15 mg/kg. Data are expressed as means ± S.E.M. ($n = 7$). Statistical test was performed at the end points of the curves. Such end points with different letters differ significantly ($p < 0.05$).

daily energy intake compared with the response following injection of vehicle alone (34.3 ± 7.6 kcal/kg day versus 8.7 ± 2.2 kcal/kg day). This corresponds to an average reduction of 75% and, as mentioned above, two individuals exhibited complete anorexia for 8 h following this type of administration. A dose of 0.1 mg/kg induced a 40% average reduction of daily energy intake, i.e. from 34.3 ± 7.6 down to 20.7 ± 3.3 kcal/kg day (Fig. 5). This reduction was also found to be statistically significant ($p < 0.05$).

3.5. *Unspecific effects*

Administration of NNC 38-1202, in the specified doses, was not associated with any visible signs of altered well-being or deviations from normal behavior, as judged by subjective surveillance.

4. Discussion

In this study, we demonstrate that administration of a histamine H_3 receptor antagonist, here exemplified with

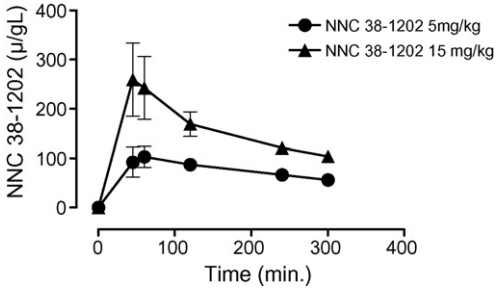

Fig. 4 – Plasma levels of NNC 38-1202 following intragastric administration of 5 ($n = 5$) and 15 ($n = 4$) mg/kg. Administrations were carried out shortly before time zero. The pigs used for these measurements constitute a subset the animals used for studies of food intake.

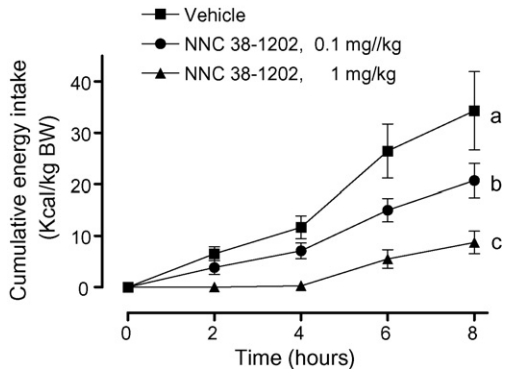

Fig. 5 – Effects of the histamine H_3 receptor antagonist NNC 38-1202 on cumulative caloric intake in obese rhesus monkeys following single subcutaneous doses of 0.1 and 1 mg/kg. Data are expressed as means ± S.E.M. (n = 9). Statistical test was performed at the end points of the curves. Such end points with different letters differ significantly ($p < 0.05$).

NNC 38-1202, effectively inhibits daily caloric intakes in pigs and in rhesus monkeys. To our knowledge, these are novel findings which are consistent with the conclusion that central histaminergic circuits are also heavily involved in the regulation of energy homeostasis in higher mammalian species. The interconnections of these circuits with other known anorectic pathways are relatively unknown (see Malmlöf et al. [16] for a recent review) and future research will hopefully reveal more details of this complex network. No neuroanatomical studies of the pig hypothalamus are to our knowledge available. However, one neuro-anatomical study of the monkey hypothalamus [17] has revealed that there is close co-localization of histaminergic nerve fibers and nerve fibers with immunoreactivity against the well-known anorectic peptide amylin [18].

We [7] and others [9] have previously shown that targeting of central histamine H_3 receptors with a selective H_3 receptor antagonist, is associated with dose-dependent decreases in food intake and body weight in rodents. In addition, we have also shown that NNC 38-1202 reduces food intake and body weight in rodents [8]. Studies performed with more unspecific H_3 antagonists like thioperamide have produced more variable results [19–21]. In association with one of our previous studies [7] we investigated the effects of an H_3 antagonist on non-specific behavioral responses in rats. We found that the H_3 antagonist increased hypothalamic histamine levels, decreased food intake and body weight but that the behavior satiety sequence (BSS) and pica behavior were unchanged. Furthermore, no sustained support of a conditioned taste aversion (CTA) could be detected. Unpublished rat data from our laboratory show that NNC 38-1202 does not stimulate pica behavior in doses up to 30 mg/kg, nor does it disrupt the BSS profile, although a certain increase in locomotor activity at a dose of 15 mg/kg (p.o.) has been observed. Further evidence that NNC 38-1202 reduces food intake in a specific manner was found in this study since no signs of non-specific reactions were observed at the actual dose levels employed. Even though these observations were of subjective nature, induction of serious unspecific effects would most probably have been detected since the normal behavioral pattern of our animals is well known to the personnel. We are also of the opinion that alterations in well-being are easier to interpret in higher mammal species than in rats.

The 98.4% identity at the amino acid level, between the monkey histamine H_3 receptor and its human counterpart found in this study confirms previously published results [13]. In addition, we show for the first time, that the pig histamine H_3 receptor has an overall amino acid identity with the human sequence of 93.3%. This number can be compared with the 93.7% identity that has been found between the rat and the human H_3 receptor [22]. Although the porcine and the rat H_3 receptors share a similar over all homology with the human counterpart they exhibit clear differences at one important site, namely in the binding pocket in amino acid positions 119 and 122. These two amino acids 119 and 122 have been shown to be critical for the pharmacological characteristics of the H_3 receptor [15]. Here the human and monkey receptor variants both have the combination T–A, while the pig expressed the combination T–V and the rat A–V. It deserves also to be mentioned the porcine receptor seems to be identical to the canine receptor in this regard, since the combination T–V is also found in dogs [15]. This means that humans, monkeys, pigs and dogs share a common amino acid, threonine, in position 119 of the H_3 receptor, whereas rats deviate from humans in both positions 119 and 122.

Since the amino acids in positions 119 and 122 are situated in the binding pocket of the H_3 receptor a given histamine H_3 receptor antagonist may have different potency on receptors originating from different species. Obviously, our functional data confirm this. The antagonist potency of NNC 38-1202 at the human and monkey H_3 receptors was found to be identical, whereas a successive decline in potency was seen from the porcine to the rat variant of the receptor.

In conclusion, the present study demonstrates that administration of a selective histamine H_3 receptor antagonist decreases food intake in pigs and primates and thus supports the idea that this therapeutic principle could be used for treatment of human obesity.

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