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Characterization of species-related differences in the pharmacology of tachykinin NK receptors 1, 2 and 3

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Short title: NK receptor pharmacology

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5 **Characterization of species-related differences in the pharmacology**
6 **of tachykinin NK receptors 1, 2 and 3.**
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43 Key words: Aprepitant; Saredutant; Talnetant; NK receptors; Species difference; NK-
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45 receptor function; Chemistry; NK-receptor sequences
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51 Abbreviations: NKR, tachykinin NK receptor; SP, Substance P; NKA, Neurokinin A;
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53 NKB, Neurokinin B; AA, amino acids; FLIPR, Fluorometric Imaging Plate Reader;
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56 TM. trans membrane
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Abstract

Tachykinin NK receptors (NKR) differ to a large degree among species with respect to their affinities for small molecule antagonists. The aims of the present study were to clone NKRs from gerbil (NK₂R and NK₃R) and dog (NK₁R, NK₂R, NK₃R) in which the sequence was previously unknown and to investigate the potency of several NKR antagonists at all known human, dog, gerbil and rat NKRs.

The NKR protein coding sequences were cloned and expressed in CHO cells. The inhibitory concentrations of selective and non-selective NKR antagonists were determined by inhibition of agonist-induced mobilization of intracellular Ca²⁺.

Receptor homology models were constructed based on the rhodopsin crystal structure to investigate and identify the antagonist binding sites and interaction points in the trans membrane (TM) regions of the NKRs.

Data collected using the cloned dog NK₁R confirmed that the dog NK₁R displays similar pharmacology as the human and the gerbil NK₁R, but differs greatly from the mouse and the rat NK₁R. Despite species-related AA differences located close to the antagonist binding pocket of the NK₂R, they did not affect the potency of the antagonists ZD6021 and saredutant. Two AA differences located close to the antagonist binding site of NK₃R likely influence the NK₃R antagonist potency, explaining the 3-10-fold decrease in potency observed for the rat NK₃R. For the first time, detailed pharmacological experiments in vitro with cloned NKRs demonstrate that not only human, but also dog and gerbil NKR displays similar antagonist pharmacology while rat diverges significantly with respect to NK₁R and NK₃R.

1. Introduction

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5 Several small molecule antagonists selective for tachykinin NK₁R, NK₂R and NK₃R
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7 are in clinical development and the selective NK₁R antagonist aprepitant is approved
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9 for treatment of emesis in response to cytostatic treatment in cancer patients [1].
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11 During the development of selective NK₁R antagonists, Beresford *et al.* discovered
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13 large discrepancies in the affinity for NK₁R from different species [2]. This led to the
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15 NK₁R family being grouped into two sub-families based on the orthologous receptor's
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17 affinity to small molecule antagonists. The first sub-family consists of the human,
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19 guinea pig, rabbit, dog, gerbil and ferret NK₁R, and the second sub-family of the rat
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21 and mouse homologues [2,3,4,5] Thus, several disease-related animal models in
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23 species other than rat or mouse have been developed for evaluation of NK₁R
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25 antagonists [6,7,8,9,10] Amino acid residues in the NK₁R responsible for species-
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27 dependent differences in antagonist pharmacology have been studied in detail,
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29 especially human Glu97, Val116 and Ser290 [5,11,12,13,14] However, although the
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31 dog appears to be an appropriate species in detecting anti-emetic effects with selective
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33 NK₁R antagonists intended for clinical use [15], the pharmacology of the dog NK₁R
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35 at the molecular level, and its homology to the human NK₁R, remain unknown to our
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37 knowledge.
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39 Consistent species-related differences in the pharmacology of NK₂R have not been
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41 reported either, although recent publications demonstrated that the selective NK₂R
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43 antagonist MEN15596 and analogues MEN14268 and MEN13918 had a marked
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45 species selectivity for inhibiting NK₂R-mediated effects in human, guinea pig and pig
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47 urinary bladder, while being 1,000-fold less potent at the rat and mouse NK₂R
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49 expressed in urinary bladder [16,17] . By contrast, species-dependent differences were
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1 not observed with MEN11420 (nepadutant) [18] or saredutant [19] Detailed site-
2 directed mutagenesis studies suggested that the Ile202 residue, located in the upper
3 part of TM5 in the human NK₂R was, at least in part, responsible for these differences
4 [16] Still, the homology between the human NK₂R and that of species commonly used
5 in tachykinin receptor pharmacology studies, such as dog and gerbil, has not been
6 reported.

7 Furthermore, species-related differences in functional response profiles between
8 human and mouse/rat have been reported for selective tachykinin NK₃R antagonists.
9 Compounds from different structural classes have 5-10-fold lower potency and 10-50-
10 fold lower affinity for rodent NK₃R compared to human counterparts [20,21] Site-
11 directed mutagenesis studies indicate that two amino acids in the second trans
12 membrane domain of the human NK₃R (Met134 and Ala146) were responsible for
13 these species differences [20,22] However, as is the case for NK₂R, the identity of
14 NK₃R from dog and gerbil remain unknown.

15 Thus, there are clearly gaps in our knowledge with respect to which species most
16 likely will predict clinical efficacy and selectivity for NK₁R, NK₂R or NK₃R
17 antagonists. Furthermore, the lack of antagonist affinity data will complicate species
18 selection for toxicological studies intended to detect adverse effects upon blocking of
19 receptor signalling. In the current study, we have cloned and sequenced the dog
20 NK₁R, NK₂R and NK₃R and the gerbil NK₂R. This should increase the understanding
21 of the molecular mechanisms underlying the species related differences in NKR
22 pharmacology, which would facilitate more relevant model system selection.
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2. Material and Methods

2.1. Chemicals

Substance P, neurokinin A and Pro⁷neurokinin B were purchased from Bachem (Peninsula Laboratories Inc, San Carlos, CA). ZD6021 was synthesized at AstraZeneca, Wilmington, USA [23]. Aprepitant [24], RP67580 [25], CP-99,994 [26], saredutant [19], talnetant [27,28] and osanetant [29,30] were synthesized at AstraZeneca Mölndal, Sweden.

2.2. Molecular cloning of the gerbil and dog NK receptors

The sequences for the human and rat NK₁R subtypes and the gerbil NK₁R have been published previously (see Table 1 for accession numbers, [5]). The receptor sequences for the gerbil NK₂R and the dog NK₁R, NK₂R and NK₃R were largely unknown and are presented in this paper and submitted to the EMBL GenBank database under the accession numbers listed in Table 2. The gerbil NK₃R sequence has been cloned and was presented in a recent study [31].

Dog hypothalamus was used as a source for cloning NK₁R and NK₃R. Dog ileum and gerbil colon were used as sources for cloning of the respective NK₂R. Total RNA was prepared from the different tissues with RNA-STAT-60 (Tel-Test Inc, Friendswood, Texas, USA). One µg of total RNA from each tissue sample was used for the first strand cDNA synthesis using SMART RACE cDNA Amplification kit (BD Biosciences, Mountain View, CA, USA). ClustalW alignment of NK₁R, NK₂R and NK₃R sequences from human, rat, mouse and guinea pig was used to select primers with high homology between different species. Primers used in the 3' RACE and in

1 the 5' RACE are listed in Table 2. The RACE fragments were characterized and
2 cloned fragments containing gerbil and dog specific NK₁R, NK₂R and NK₃R
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4 sequences spanning the open reading frame were identified.
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7 Complementary DNA (2.5 µl) from the harvested tissues indicated above was used in
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9 the optimized full-length PCR with forward and reverse primers (20 µM of each),
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11 1xPCR buffer, 5 mM of each dNTP and 1 U Pfu Ultra (Stratagene, La Jolla, CA,
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13 USA). A Kozak sequence (GCCACC) was introduced before the ATG in each
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15 construct. Conditions were optimized for each primer pair used. The resulting PCR
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17 products for the gerbil and dog NK₁R and NK₂R were cloned into pIRESHyg2
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19 expression vector (Clontech, Palo Alto, CA, USA). The full-length cDNA of dog and
20
21 gerbil NK₃ receptor was cloned into pCDNA/FRT expression vector (Invitrogen,
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23 Carlsbad, CA, USA). In order to construct a full length clone of dog NK₃R, the 5'-end
24
25 of the dog NK₃R was cloned using genomic sequence data (TI number 356163905)
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27 from a trace file as a template for PCR reactions.
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34 Multiple sequence alignments were constructed using ClustalX version 2.0 [32], and
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36 the TM domains were predicted using the TMHMM server version 2.0
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38 (<http://www.cbs.dtu.dk/services/TMHMM/>). A position in the alignments is
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40 considered TM if the majority of the individual sequences are predicted to be TM at
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42 that position.
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46 2.3. Cell culture and transfection

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48 Chinese Hamster Ovary (CHO) cells (ATCC, Middlesex, UK) or CHO-FlpIN cells
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50 (Invitrogen, Carlsbad, CA, USA) were transfected with the different constructs. All
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52 accession numbers for the sequences used to transfect the CHO cells are listed in table
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2. NK₁₋₃ R-containing clones were selected by growth in appropriate selection media and tested for functionality in a Ca²⁺ mobilization assay.

CHO cells stably expressing human NK₁R was supplied by AstraZeneca R&D, Wilmington, USA and human NK₂R, rat NK₁R, rat NK₂R and rat NK₃R were transfected in house (see Table 1 for gene accession numbers). Stable transfectants were maintained by supplementing culture media (NutMix F12 (HAM) with Glutamax I and 10% FBS) (Invitrogen, Carlsbad, CA, USA) with suitable selection depending on the expressing vector. Cultures were kept at 37°C in a 5% CO₂-incubator and routinely passaged when 70-80% confluent for up to 20-25 passages.

2.4. Calcium mobilization assay

Ca²⁺ mobilization was studied in CHO cells or in CHO FlpIn cells stably expressing human, rat, gerbil or dog NK1-3 receptors using the cytoplasmic Ca_i²⁺ indicator Fluo-4 (TEFLABS 0152, Austin, TX, USA). Cells were seeded into black-walled clear-base 96-well plates (Costar, #3904) at a density of 35,000 cells per well in culture media and grown for 24 h in a 37°C CO₂-incubator. The cells were incubated with 4 μM of Fluo-4 (TEFLABS 0152) in loading media (Nut Mix F12 (HAM), glutamax I, 22 mM HEPES, 2.5 mM probenecid (P-8761, Sigma, St. Louis, MO, USA) and 0.04 % pluronic F-127 (P-2443 Sigma, St. Louis., MO, USA) for 30 minutes in a 37°C CO₂-incubator. The Fluo-4-loaded cells were then washed three times in assay buffer (Hanks Balanced Salt Solution, 20 mM HEPES, 2.5 mM probenecid and 0.1% BSA). The plates were placed into the Fluorometric Imaging Plate Reader (FLIPR™) to monitor cell fluorescence (λ_{ex} = 488nm, λ_{em} = 540nm) before and after the addition of antagonists and/or agonists. Antagonists and agonists were dissolved in assay buffer (final DMSO, D2650, Sigma. concentration kept below 1%) in 96-well plates

1 and added to the loaded cells by the automated pipettor in the FLIPR™. Loaded cells
2 were pre-incubated with antagonists for 2 minutes before addition of agonist (0.08 nM
3 SP for NK₁R, 0.15 nM NKA for NK₂R and 1.0 nM pro7NKB for NK₃R). Ca_i²⁺
4 responses were measured as peak fluorescence intensity minus basal fluorescence
5 after agonist addition.
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14 2.6. *Rhodopsin homology model*

15 The sequences of the human NK₁₋₃R were aligned to the bovine rhodopsin and a
16 subset of rhodopsin sequences, covering different degrees of sequence identity. The
17 pair wise alignments of NK₁₋₃R and rhodopsin, were extracted and adjusted manually
18 to optimize compatibility with structure and frequently occurring sequence motifs
19 amongst GPCRs. The rhodopsin-NK₁₋₃R alignment was used as input for the
20 automatic modeling and used for the evaluation of the WhatCheck module [33]. All
21 binding site results and discussions are based on predictions drawn from NKR
22 modified rohdopsin homology model. A limitation of the Whatif modeling tool is that
23 insertions and gaps are not considered. In this case, however, the active site should not
24 be affected by this limitation.
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44 2.7. *Data analysis*

45 The calcium mobilization data generated *in vitro* were fitted to a four parameter
46 equation using Excel Fit. IC₅₀s for antagonists were determined from concentration-
47 response curves for each compound. Potency (K_B)-values for antagonists were
48 calculated using the Cheng-Prusoff equation [34] and expressed as pK_B-values (pK_B =
49 -log K_B). All data are expressed as Mean ± S.E.M.
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3. Results

3.1. Sequence characterization and antagonist pharmacology of the tachykinin NK₁ receptor

Multiple sequence alignments of the gerbil, human, dog, rat, mouse and guinea pig NK₁R are shown in Fig. 1A. Unlike rat and gerbil, the dog NK₁R displays 100% homology with human in the trans-membrane regions. Thus, the Val116 and Ile290 residues, previously identified as important for antagonist binding, are identical between the dog and the human NK₁ receptor sequences. Also extracellular residues such as Glu97 have also been suggested as being responsible for species selectivity of antagonists. Indeed, gerbil and dog NK₁R also contained a Glu residue in this position while rat and mouse counterparts contained Val97.

The selective NK₁R antagonists aprepitant (Fig. 1B, Table 3), CP99,994 (Table 3), and the pan-NK antagonist ZD6021 (Table 3) were slightly more potent (5-10 fold) inhibitors of the SP-induced responses in cells expressing the dog compared to the human NK₁R. RP67580 displayed similar potency at NK₁R from all species evaluated (Table 3).

Fig 1C illustrates a rhodopsin-NK₁R homology model which emphasizes the role of AA 116 and 290 in dictating species differences in NK₁R antagonist pharmacology. The polar 290Ser in rat is smaller and less hydrophobic compared to the non-polar 290Ile present in human and dog NK₁R. In contrast, the Leu116Val (rat/human) is a rather conservative AA exchange. There are no other species-related differences in any of the other residues found to be close to the aprepitant binding site using this model (Fig 1C).

3.2. Sequence characterization and antagonist pharmacology at the tachykinin NK₂ receptor

The alignments of the gerbil, human, dog, rat, mouse and guinea pig NK₂Rs, are shown in Fig. 2A. The residues located in the proposed saredutant binding site differ between species. Position 202 located within TM 5 is an Ile in the human and dog NK₂R while the gerbil and rat NK₂R have a Phe in this position. Position 205, also located in TM 5, is Ile in human, rat and dog NK₂R and Val in gerbil NK₂R, while position 267 (located in TM6) has Leu in the human, rat and gerbil NK₂R, which is replaced by a Phe in the dog NK₂R.

Despite the interspecies differences in AA in TM regions, the selective NK₂R antagonist saredutant (Fig. 2B, Table 3), and the pan-NKR antagonist ZD6021 (Table 3), had a similar potency at the NK₂R from the various species.

Fig. 2C illustrates the chemical structures of AA that differ between species and that are presumed to be involved in NK₂R binding of saredutant. All the differences between AA in the NK₂R binding site contain neutral, nonpolar AA. Even though there are differences in the hydrophobicity among residues, they do not seem to affect the potency of the tested NK₂R antagonists (Table 3).

3.3. Sequence characterization and antagonist pharmacology at the tachykinin NK₃ receptor

Alignments of the gerbil, human, dog, rat, mouse and guinea pig NK₃R sequences are shown in Fig. 3A. Key residues important for the binding of talnetant (Met134, Ala146 and Ile317) are identical between gerbil, dog and human, while the corresponding residues in the rat and mouse sequences are Val134, Gly146 and Val317 respectively.

1 The selective NK₃R antagonists talnetant (Fig. 3B and Table 3) and osanetant (Table
2 3) had similar potency at human, dog and gerbil NK₃R while being approximately 10-
3 4 fold less potent for the rat NK₃R. Furthermore, the pan-NKR antagonist ZD6021 also
5 6 displayed 10-fold higher potency at human, dog and gerbil NK₃R compared to rat
7 8 NK₃R (Table 3).
9

10 Fig. 3C illustrates the rhodopsin NK₃R homology model which emphasizes the role of
11 12 key residues for talnetant binding. The human NK₃R positions 265 and 306 differ
13 14 from all other species studied. The dog NK₃R has a Met at position 202 while the
15 16 other species have an Ile. Nevertheless, based on the pharmacological profile of
17 18 antagonists at NK₃R for the various species it appears as if the rat/mouse-specific AA
19 20 at residues 134, 146 and 317 may play a role in the lower potency of NK₃R
21 22 antagonists at rat NK₃R.
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32 **4. Discussion**

33 It has been known for quite some time that there are major differences in the affinity
34 35 of many selective NK₁R antagonists between human and rat/mouse NK₁R. Therefore,
36 37 since these commonly used experimental animals are not always suitable for
38 39 evaluation of clinically relevant compounds, other species have been used instead,
40 41 including gerbils and dogs. Indeed, gerbils have been used for detecting potential
42 43 behavioural [6] anti-depressive [9], visceral anti-hyperalgesic [10] and gut motility
44 45 [7] effects of NK₁R antagonists. We recently showed that the gerbil NK₁R shares key
46 47 residues (Glu97, Val116, Ile290) with the human NK₁R homologue and that it has
48 49 similar affinity for several known NK₁R antagonists [5] (Fig 1C). Dogs are often used
50 51 to study the efficacy of anti-emetic compounds like NK₁R antagonists [15]. The
52 53 current study extends our previous findings in that the dog NK₁R also contains the
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1 same important residues for antagonist function as human and gerbil NK₁R. This
2 provides confirmation at the molecular level that the dog belongs to the human-like
3 NK₁R sub-family which has been previously suggested based on experiments *in vivo*
4 [35]. Interestingly, the compounds ZD6021, CP99,994 and aprepitant were actually
5 slightly more potent (5-10-fold) at dog NK₁R compared to gerbil and man. The reason
6 for this is unclear since the receptors display 100% homology in the TM region.
7 RP67580, often referred to as a rat-selective NK₁R antagonist, was equipotent at dog,
8 rat and man. Hence, the rat-selectivity is not supported in the current study and is in-
9 line with previous data [5]. Aprepitant is clearly less potent at rat NK₁R than at NK₁R
10 from other species. However, based on our data, aprepitant could be useful as a tool
11 compound in rat models since it displays similar potency as RP67580, an NK₁R
12 antagonist commonly used in rat models, but appears to have better DMPK properties
13 such as CNS penetration and metabolic half-life [36].
14 Cloning and sequencing of gerbil and dog NK₂R provides data for a more thorough
15 comparison between species. In contrast to NK₁R, rather large discrepancies were
16 found in the TM region in the NK₂R. Residues 202, 205 and 267 had a variety of
17 amino acids in this position among species (Fig 2C). Interestingly, all three of these
18 residues have been implicated in the binding of NK₂R antagonists [37]. Despite the
19 potential importance of these residues for binding and the rather large degree of
20 variation between species, we were unable to identify significant differences of the
21 potency of saredutant and ZD6021 at NK₂R among the species studied. However, it
22 can not be excluded that the potency of structurally different compounds could have
23 different potency at NK₂R from gerbil and dog [16].
24 Consistent with previous findings by [38], both talnetant and osanetant displayed
25 about a 5-10-fold lower potency at the rat NK₃R compared to the human NK₃R.
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1 ZD6021 also displayed a relatively weak potency for rat NK₃R (30-fold weaker than
2 at human NK₃R). The current study extends these findings demonstrating that the
3 antagonists tested have similar potency at dog and gerbil NK₃R as to human NK₃R.
4 Recent data show that talnetant and osanetant interact within overlapping but not
5 identical binding pockets in the human tachykinin NK₃R trans membrane domains
6 [39]. The human-like pharmacology of gerbil and dog NK₃R is consistent with the
7 presence of a methionine located in position 134, a residue that has previously been
8 shown to be importance for talnetant and osanetant binding to the human NK₃R (Fig
9 3C). The Ile317Val AA change may induce less sterical hindrance, and may affect the
10 functional potency of the tested compounds (Fig 3C). The above suggestions support
11 that gerbils and dog represent appropriate species for evaluating the efficacy of
12 tachykinin NK₃R antagonists intended for clinical use. Indeed, osanetant has
13 demonstrated anxiolytic-like and antidepressant-like effects in gerbils [40].
14 Single nucleotide polymorphisms in genes encoding receptors can affect many aspects
15 of receptor function and antagonist binding. For instance, four variants of the human
16 NK₂R are common within the human population [41]. The current study utilized the
17 Thr23_Arg375 variant, however saredutant and ZD6021 display similar potency at all
18 four human variants. If AA-exchanging polymorphisms in NKRs occur within or
19 between strains of the experimental animals compared here is unknown to our
20 knowledge.
21 Given the above species difference in NKR pharmacology the study of receptor-
22 mediated toxicology of any NKR modulator, both agonist and antagonists, is not
23 trivial. Ideally, for such studies to be meaningful, selected species for toxicology
24 testing should express the receptor in a similar way and have similar functions as in
25 humans. Concomitantly, selected species should be well characterized with historical
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1 data, in order to differentiate any treatment related lesions from spontaneous
2 (background) pathology. For these reasons, studies of potential toxicology of NK₂R
3 antagonists seem to be appropriately conducted in well-known species such as the rat
4 and dog, while dogs appear to suffice for detected potential unwanted effects of NK₁R
5 and NK₃R antagonism.
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Table 1. Accession numbers for the cloned NKR from human, rat, gerbil and dog and the accession numbers for NKR from mouse and guinea pig used in the alignments of NK receptors.

Receptor	Species	Acc number
NK ₁	human	NM_001058
NK ₂	human	AY322545
NK ₃	human	M89473
NK ₁	rat	J05097
NK ₂	rat	M31838
NK ₃	rat	NM_017053
NK ₁	gerbil	AJ884917
NK ₂	gerbil	AJ884918
NK ₃	gerbil	AM157740
NK ₁	dog	AJ884915
NK ₂	dog	AJ884916
NK ₃	dog	AM423140
NK ₁	mouse	NM_009313
NK ₂	mouse	NM_009314
NK ₃	mouse	NM_021382
NK ₁	guinea pig	P30547
NK ₂	guinea pig	Q64077
NK ₃	guinea pig	P30098

Table 2. Primers used in the 3'RACE and in the 5'RACE of NK₁R, NK₂R and NK₃R.

receptor	species	primer sequence	reaction	original sequence
NK1	dog	CCCTCGTAGTCGCCGGCGCTGATAAAG	5'RACE	dog S75109
NK1	dog	CCCTTTATCAGCGCCGGCGACTACGAG	3'RACE	dog S75109
NK2	dog	CACTGTAGGCGACGATCATCACCAAGAG	5'RACE	dog S75024
NK2	dog	TCTCTTGGTGATGATCGTCGCCTACAGTG	3'RACE	dogS75024
NK3	dog	GGGACCTTCTGGCCATTGCACATAACA	5'RACE	dog S75029
NK3	dog	CATGCCAGGCCGTACCCTTTGTTATGTGC	3'RACE	dog S75029
NK2	gerbil	GGAAAGCAAGCCGGAATCCAGAGCG	5'RACE	rat and mouse alignment
NK2	gerbil	GGCTGCCCTACCACCTCTACTTCATCCT	3'RACE	gerbil

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Table 3. Potency of selective NK receptor antagonists and the pan-NKR antagonist ZD6021 on substance P (NK₁R), NKA (NK₂R) and Pro7NKB (NK₃R) evoked increases in intracellular Ca²⁺ mobilization. Data are expressed as pK_B values ± SEM, n = 3-5.

Compound	Human	Dog	Gerbil	Rat
NK₁R				
ZD6021	8.6 ± 0.4	9.5 ± 0.2	9.0 ± 0.2	<6
RP67580	7.1 ± 0.4	7.1 ± 0.6	6.5 ± 0.2	7.3 ± 0.4
CP99,994	8.7 ± 0.2	9.8 ± 0.3	8.9 ± 0.3	5.9 ± 0.2
Aprepitant	8.7 ± 0.2	9.2 ± 0.1	8.8 ± 0.2	7.3 ± 0.1
NK₂R				
ZD6021	8.3 ± 0.4	8.4 ± 0.2	8.4 ± 0.3	8.1 ± 0.1
Saredutant	9.1	9.4 ± 0.1	9.3 ± 0.2	9.4 ± 0.1
NK₃R				
ZD6021	7.9 ± 0.3	7.8 ± 0.1	7.9 ± 0.1	6.7 ± 0.2
Talnetant	8.6 ± 0.3	8.4 ± 0.2	8.4 ± 0.1	7.4 ± 0.2
Osanetant	8.4 ± 0.5	8.2 ± 0.2	8.0 ± 0.2	7.4 ± 0.3

Legends to Figures

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5 Figure 1. (A): Sequence alignments of NK₁R from gerbil, human, dog, rat, mouse
6
7 and guinea pig. TM regions are under lined, sequence differences indicated by gray
8
9 shading and amino acids predicted to be important for antagonist binding are boxed.
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11 (B): Dose-dependent inhibitory effect of the selective NK₁R antagonist aprepitant on
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13 SP-evoked mobilization of intracellular Ca²⁺ in cells expressing the NK₁R from
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15 various species. Representative curves from each animal from three experiments
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17 done, are shown, all performed in the same experiment (see Table 3). (C): Chemical
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19 sequences of amino acids interacting in the NK₁R binding site in the presence of
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21 aprepitant. Bold numbers on AA indicate change as compared to the human AA. AA
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23 marked in bold show a TM region species difference.
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32 Figure 2. (A): Sequence alignments of NK₂R from gerbil, human, dog, rat, mouse
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34 and guinea pig. Tm regions are under lined, sequence differences indicated by gray
35
36 shading and amino acids predicted to be important for antagonist binding are boxed.
37
38 (B): Representative curve illustrating the dose-dependent inhibitory effect of the
39
40 selective NK₂ receptor antagonist saredutant on NKA-evoked mobilization of
41
42 intracellular Ca²⁺ in cells expressing the NK₂ receptor from various species.
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44 Representative curves from each animal from three experiments done, are shown, all
45
46 performed in the same experiment (see Table 3). (C): Chemical sequences of amino
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48 acids interacting in the NK₂R binding site in the presence of saredutant. Bold numbers
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50 on AA indicate change as compared to the human AA. AA marked in bold show a
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52 TM region species difference.
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5 Figure 3. (A): Sequence alignments of NK₃Rs from gerbil, human, dog, rat, mouse
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7 and guinea pig. Tm regions are under lined, sequence differences indicated by gray
8
9 shading and amino acids predicted to be important for antagonist binding are boxed.
10
11 (B): Representative curve illustrating the dose-dependent inhibitory effect of the
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13 selective NK₃R antagonist talnetant on NKB-evoked mobilization of intracellular Ca²⁺
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15 in cells expressing the NK₃R from various species. Representative curves from each
16
17 animal from three experiments done, are shown, all performed in the same experiment
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19 (See Table 3). (C): Chemical sequences of amino acids interacting in the NK₃R
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21 binding site in the presence of talnetant. Bold numbers on AA indicate change as
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23 compared to the human AA. AA marked in bold show a TM region species
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25 difference.
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Fig 1A

1	1	MDNVLPQSDSLFPNISTNSSESQFVQPAWQIVLWAAAYTVIVVTSVWGNVVMWIIILAHKFRMRTVTNYF	gerbil NK1r
2	1	MDNVLPVDSDLSPNISTNTSEENQFVQPAWQIVLWAAAYTVIVVTSVWGNVVMWIIILAHKFRMRTVTNYF	human NK1r
3	1	MDNVLPVDSDLFPNISTNTSEENQFVQPAWQIVLWAAAYTVIVVTSVWGNVVMWIIILAHKFRMRTVTNYF	dog NK1r
4	1	MDNVLPVDSDLFPNISTNTSESNQFVQPTWQIVLWAAAYTVIVVTSVWGNVVMWIIILAHKFRMRTVTNYF	rat NK1r
5	1	MDNVLPVDSDLFPNISTNTSEENQFVQPAWQIVLWAAAYTVIVVTSVWGNVVMWIIILAHKFRMRTVTNYF	mouse NK1r
6	1	MDNVLPVDSDLFPNISTNTSEENQFVQPAWQIVLWAAAYTVIVVTSVWGNVVMWIIILAHKFRMRTVTNYF	guinea pig NK1r
7		TM1	
8	71	LVNLAFAEASMAAFNTVFNFTYAVHNEWYGLFYCKFHNFFPIAIFASISMTAVAFDRYMAIIHPLQP	gerbil NK1r
9	71	LVNLAFAEASMAAFNTVFNFTYAVHNEWYGLFYCKFHNFFPIAIFASISMTAVAFDRYMAIIHPLQP	human NK1r
10	71	LVNLAFAEASMAAFNTVFNFTYAVHNEWYGLFYCKFHNFFPIAIFASISMTAVAFDRYMAIIHPLQP	dog NK1r
11	71	LVNLAFAEASMAAFNTVFNFTYAVHNEWYGLFYCKFHNFFPIAIFASISMTAVAFDRYMAIIHPLQP	rat NK1r
12	71	LVNLAFAEASMAAFNTVFNFTYAVHNEWYGLFYCKFHNFFPIAIFASISMTAVAFDRYMAIIHPLQP	mouse NK1r
13	71	LVNLAFAEASMAAFNTVFNFTYAVHNEWYGLFYCKFHNFFPIAIFASISMTAVAFDRYMAIIHPLQP	guinea pig NK1r
14		TM2	
15		TM3	
16	141	RLSATATKVVIFVINVLALLLAFPGQYSTTETMPGRVVCMIENPEHPNRTYEKAYHICVTVLIFFLPLL	gerbil NK1r
17	141	RLSATATKVVIFVINVLALLLAFPGQYSTTETMPGRVVCMIENPEHPNRTYEKAYHICVTVLIFFLPLL	human NK1r
18	141	RLSATATKVVIFVINVLALLLAFPGQYSTTETMPGRVVCMIENPEHPNRTYEKAYHICVTVLIFFLPLL	dog NK1r
19	141	RLSATATKVVIFVINVLALLLAFPGQYSTTETMPGRVVCMIENPEHPNRTYEKAYHICVTVLIFFLPLL	rat NK1r
20	141	RLSATATKVVIFVINVLALLLAFPGQYSTTETMPGRVVCMIENPEHPNRTYEKAYHICVTVLIFFLPLL	mouse NK1r
21	141	RLSATATKVVIFVINVLALLLAFPGQYSTTETMPGRVVCMIENPEHPNRTYEKAYHICVTVLIFFLPLL	guinea pig NK1r
22		TM4	
23	211	MIGYAYTVVGIITLWASEIPGDSSDRYHEQVSAKRKVVQMMIVVCTFAICWLPFHIFELLFYINEDLYLK	gerbil NK1r
24	211	MIGYAYTVVGIITLWASEIPGDSSDRYHEQVSAKRKVVQMMIVVCTFAICWLPFHIFELLFYINEDLYLK	human NK1r
25	211	MIGYAYTVVGIITLWASEIPGDSSDRYHEQVSAKRKVVQMMIVVCTFAICWLPFHIFELLFYINEDLYLK	dog NK1r
26	211	MIGYAYTVVGIITLWASEIPGDSSDRYHEQVSAKRKVVQMMIVVCTFAICWLPFHIFELLFYINEDLYLK	rat NK1r
27	211	MIGYAYTVVGIITLWASEIPGDSSDRYHEQVSAKRKVVQMMIVVCTFAICWLPFHIFELLFYINEDLYLK	mouse NK1r
28	211	MIGYAYTVVGIITLWASEIPGDSSDRYHEQVSAKRKVVQMMIVVCTFAICWLPFHIFELLFYINEDLYLK	guinea pig NK1r
29		TM5	
30		TM6	
31	281	KFIQQVYLAIMWLAMSSTMYNPIIYCCLNDRFRLGFKHAFRCPPFISAGDYEGLMKSTRYLQTQGSVYK	gerbil NK1r
32	281	KFIQQVYLAIMWLAMSSTMYNPIIYCCLNDRFRLGFKHAFRCPPFISAGDYEGLMKSTRYLQTQGSVYK	human NK1r
33	281	KFIQQVYLAIMWLAMSSTMYNPIIYCCLNDRFRLGFKHAFRCPPFISAGDYEGLMKSTRYLQTQGSVYK	dog NK1r
34	281	KFIQQVYLAIMWLAMSSTMYNPIIYCCLNDRFRLGFKHAFRCPPFISAGDYEGLMKSTRYLQTQGSVYK	rat NK1r
35	281	KFIQQVYLAIMWLAMSSTMYNPIIYCCLNDRFRLGFKHAFRCPPFISAGDYEGLMKSTRYLQTQGSVYK	mouse NK1r
36	281	KFIQQVYLAIMWLAMSSTMYNPIIYCCLNDRFRLGFKHAFRCPPFISAGDYEGLMKSTRYLQTQGSVYK	guinea pig NK1r
37		TM7	
38	351	VSRLETTISTVVGAEHEEPEEGPKATPSSLDLTSNGSSRSNSKTMTESSSFYSNMLA	gerbil NK1r
39	351	VSRLETTISTVVGAEHEEPEEGPKATPSSLDLTSNGSSRSNSKTMTESSSFYSNMLA	human NK1r
40	351	VSRLETTISTVVGAEHEEPEEGPKATPSSLDLTSNGSSRSNSKTMTESSSFYSNMLA	dog NK1r
41	351	VSRLETTISTVVGAEHEEPEEGPKATPSSLDLTSNGSSRSNSKTMTESSSFYSNMLA	rat NK1r
42	351	VSRLETTISTVVGAEHEEPEEGPKATPSSLDLTSNGSSRSNSKTMTESSSFYSNMLA	mouse NK1r
43	351	VSRLETTISTVVGAEHEEPEEGPKATPSSLDLTSNGSSRSNSKTMTESSSFYSNMLA	guinea pig NK1r

Fig 1B

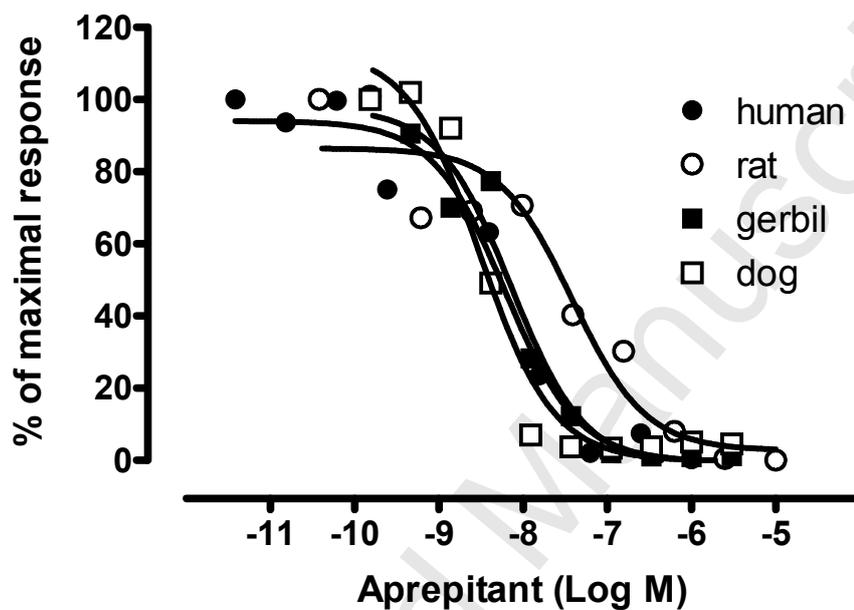
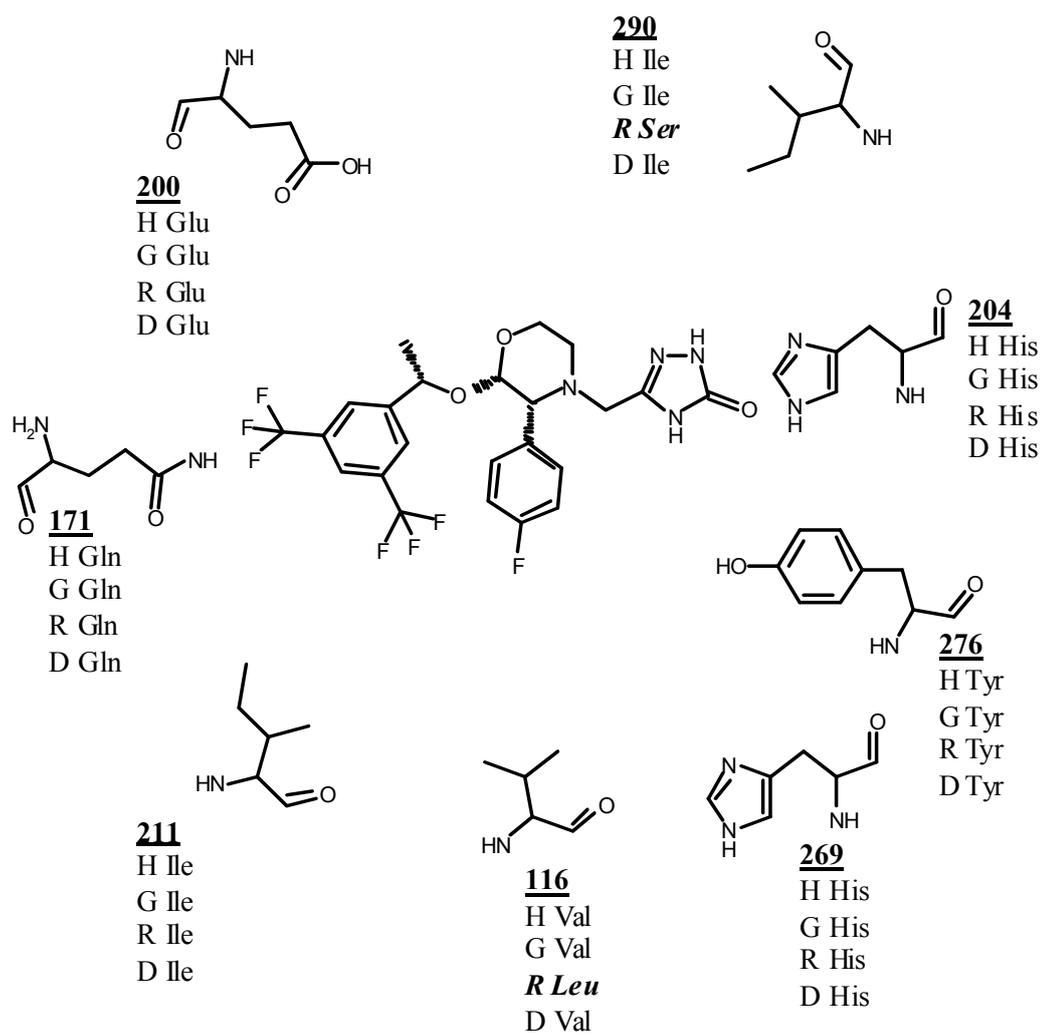


Fig 1C

Species difference in NK1R.

Aprepitant

Fig 2A

1	MGAYAIIVTDNIIISGLQGNNTTGVTAFSMPAWQLALNATAYLALVAVTGNATVIWIIIAHERMRTVTNY	gerbil NK2r
2	1	human NK2r
3	1	dog NK2r
4	1	rat NK2r
5	1	mouse NK2r
6	1	guinea pig NK2r
7	1	
8		
9		
10	71	gerbil NK2r
11	71	human NK2r
12	71	dog NK2r
13	71	rat NK2r
14	71	mouse NK2r
15	71	guinea pig NK2r
16		
17		
18		
19		
20		
21	141	gerbil NK2r
22	141	human NK2r
23	141	dog NK2r
24	141	rat NK2r
25	141	mouse NK2r
26	141	guinea pig NK2r
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Fig 2B

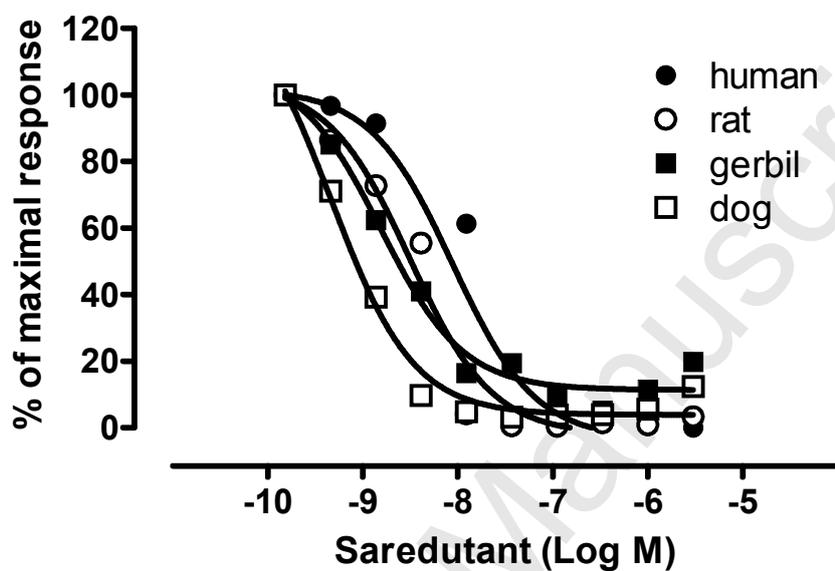


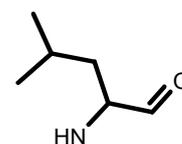
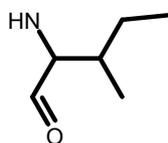
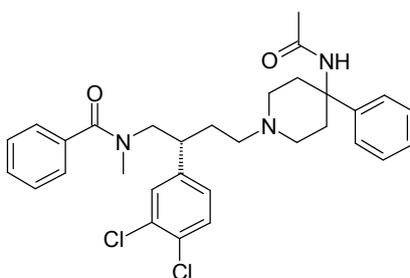
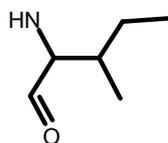
Fig 2C

Species difference in NK2R202

H Ile

G Phe***R Phe***

D Ile

267

H Leu

G Leu

R Leu

D Phe205

H Ile

G Val

R Ile

D Ile

SR48968

Fig 3A

1	MASVPTAESESWTDGAAGVGTQVGNLSSALGV-----TEWFALQ--AGNFSSALG---VPVTSPTPS	gerbil NK3r
1	MATEPAAET--WIDGGGGVGDADAVNLTAASLAAGAATGAVETGHLQLLDQAGNESSSPFAALGLFVSPAPS	human NK3r
1	MGSLPAAAT--WDRAGPAGAGGGNLSAALAGAAM--AAGAEWLQLLGRAGNLSASP----PAASAAPA	dog NK3r
1	MASVPRGEN--WTDGTVEVGTHTGNLSSALGV-----TEWLALQ--AGNFSSALG---LPATTQAPS	rat NK3r
1	MASVPTGEN--WTDGTAGVGSHTGNLSSALGI-----TEWLALQ--AGNFSSALG---LPVTSQAPS	mouse NK3r
1	MASPAGNLS- AWPGHWPPPAALNLTSSAP ----- TASPS ---- PAPSWTPS	guinea pig NK3r
58	QARANLTNQFVQPSWRIALWSLAYGLVVAVAVFGNLIIVIMIILAHKRMRTVTINYFLVNLAFLSDASMAAFN	gerbil NK3r
69	QFRANLTNQFVQPSWRIALWSLAYGLVVAVAVFGNLIIVIMIILAHKRMRTVTINYFLVNLAFLSDASMAAFN	human NK3r
63	QFRANLTNQFVQPSWRIALWSLAYGLVVAVAVFGNLIIVIMIILAHKRMRTVTINYFLVNLAFLSDASMAAFN	dog NK3r
56	QVRANLTNQFVQPSWRIALWSLAYGLVVAVAVFGNLIIVIMIILAHKRMRTVTINYFLVNLAFLSDASMAAFN	rat NK3r
56	QVRANLTNQFVQPSWRIALWSLAYGLVVAVAVFGNLIIVIMIILAHKRMRTVTINYFLVNLAFLSDASMAAFN	mouse NK3r
45	- PRGPAHFPLQPPWRVALWSLAYGAVVAVAVFGNLIIVIMIILAHKRMRTVTIN SFLVNLAFLADAMAAEN	guinea pig NK3r
	TM1	TM2
128	TLVNFYIALHSEWYFGANYCRFQNFPPITAVFAS IYSMTAIAVDRYMAIIDPLKPRLSATATKIVIGSIW	gerbil NK3r
139	TLVNFYIALHSEWYFGANYCRFQNFPPITAVFAS IYSMTAIAVDRYMAIIDPLKPRLSATATKIVIGSIW	human NK3r
133	TLVNFYIALHSEWYFGANYCRFQNFPPITAVFAS IYSMTAIAVDRYMAIIDPLKPRLSATATKIVIGSIW	dog NK3r
126	TLVNFYIALHSEWYFGANYCRFQNFPPITAVFAS IYSMTAIAVDRYMAIIDPLKPRLSATATKIVIGSIW	rat NK3r
126	TLVNFYIALHSEWYFGANYCRFQNFPPITAVFAS IYSMTAIAVDRYMAIIDPLKPRLSATATKIVIGSIW	mouse NK3r
114	ALVNFYIALHSEWYFGANYCRFQNFPPITAVFAS IYSMTAIAVDRYMAIIDPLKPRLSATATKIVIGSIW	guinea pig NK3r
	TM3	
198	ILAFLLAFQCLYSKIKVMPGRTLQCYVQWPEGPKQHFTYHIIVIIILVYCFPLLIMCVITYTIVGITLWGGE	gerbil NK3r
209	ILAFLLAFQCLYSKIKVMPGRTLQCYVQWPEGPKQHFTYHIIVIIILVYCFPLLIMCVITYTIVGITLWGGE	human NK3r
203	ILAFLLAFQCLYSKIKVMPGRTLQCYVQWPEGPKQHFTYHIIVIIILVYCFPLLIMCVITYTIVGITLWGGE	dog NK3r
196	ILAFLLAFQCLYSKIKVMPGRTLQCYVQWPEGPKQHFTYHIIVIIILVYCFPLLIMCVITYTIVGITLWGGE	rat NK3r
196	ILAFLLAFQCLYSKIKVMPGRTLQCYVQWPEGPKQHFTYHIIVIIILVYCFPLLIMCVITYTIVGITLWGGE	mouse NK3r
184	ILAFLLAFQCLYSKIKVMPGRTLQCYVQWPEGPKQHFTYHIIVIIILVYCFPLLIMCVITYTIVGITLWGGE	guinea pig NK3r
	TM4	TM5
268	IPGDTCDKYHEQLKAKRKKVVKMMIIVVMTFAICWLPYHIFILTAIYQQLNRNKYIQQVYLASFWMAMSS	gerbil NK3r
279	IPGDTCDKYHEQLKAKRKKVVKMMIIVVMTFAICWLPYHIFILTAIYQQLNRNKYIQQVYLASFWMAMSS	human NK3r
273	IPGDTCDKYHEQLKAKRKKVVKMMIIVVMTFAICWLPYHIFILTAIYQQLNRNKYIQQVYLASFWMAMSS	dog NK3r
266	IPGDTCDKYHEQLKAKRKKVVKMMIIVVMTFAICWLPYHIFILTAIYQQLNRNKYIQQVYLASFWMAMSS	rat NK3r
266	IPGDTCDKYHEQLKAKRKKVVKMMIIVVMTFAICWLPYHIFILTAIYQQLNRNKYIQQVYLASFWMAMSS	mouse NK3r
254	IPGDTCDKYHEQLKAKRKKVVKMMIIVVMTFAICWLPYHIFILTAIYQQLNRNKYIQQVYLASFWMAMSS	guinea pig NK3r
	TM6	
338	TMYNPIIYOCNLNKRFRAGFKRAFRCPPFIQVSSYDELELKTTRFHPTRQSSLYTVSRMESVTVLFDPNDD	gerbil NK3r
349	TMYNPIIYOCNLNKRFRAGFKRAFRCPPFIQVSSYDELELKTTRFHPTRQSSMYTVSRMESMTVWFDPNDA	human NK3r
343	TMYNPIIYOCNLNKRFRAGFKRAFRCPPFIQVSSYDELELKTTRFHPTRQSSLYTVSRMESMTVWFDPNDA	dog NK3r
336	TMYNPIIYOCNLNKRFRAGFKRAFRCPPFIQVSSYDELELKTTRFHPTRQSSLYTVSRMESVTVLFDPNDD	rat NK3r
336	TMYNPIIYOCNLNKRFRAGFKRAFRCPPFIQVSSYDELELKTTRFHPTRQSSLYTVSRMESVTVLFDPNDD	mouse NK3r
324	TMYNPIIYOCNLNKRFRAGFKRAFRCPPFIQVSSYDELELKTTRFHPTRQSSLYTVSRMESMTVWFDPNDD	guinea pig NK3r
	TM7	
408	DPAKSSRKKRAVPRDPSANGCSRRRNSKSASTSSFISSPYTSVDEYS	gerbil NK3r
419	DITRSSRKKRAVPRDPSANGCSRRRNSKSASTSSFISSPYTSVDEYS	human NK3r
413	DNTKSSRKKRAVPRDPSANGCSRRRNSKSASTSSFISSPYTSVDEYS	dog NK3r
406	DFTKSSRKKRAVPRDPSANGCSRRRNSKSASTSSFISSPYTSVDEYS	rat NK3r
406	DPAKSSRKKRAVPRDPSANGCSRRRNSKSASTSSFISSPYTSVDEYS	mouse NK3r
394	DSARSSRKKRAVPRDPSANGCSRRRNSKSASTSSFISSPYTSVDEYS	guinea pig NK3r

Fig 3B

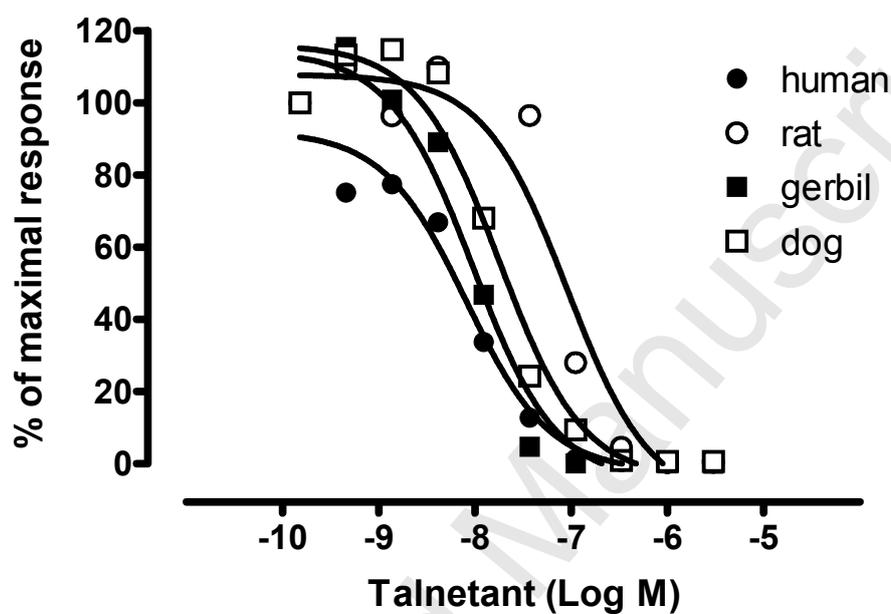
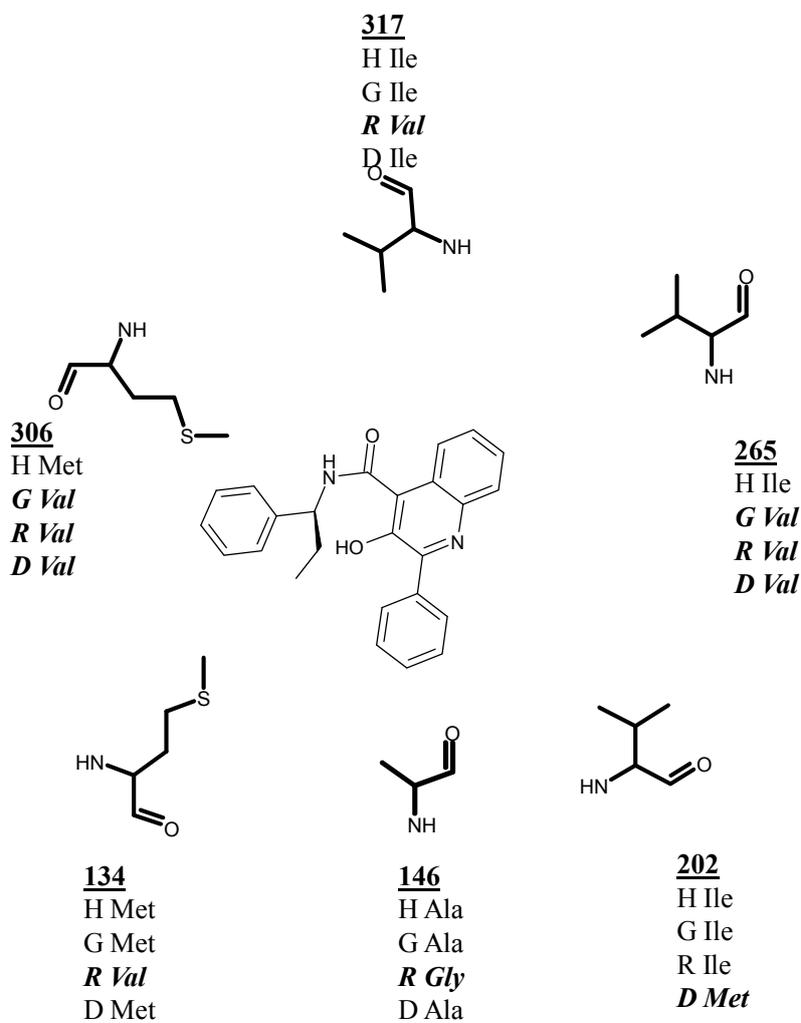


Fig 3C

Species difference in NK3R

Talnetant

Experiments with Neurokinin receptors (NKR) demonstrate that not only human, but also dog and gerbil NKR displays similar antagonist pharmacology while rat diverges significantly with respect to NK₁R and NK₃R.

Potencies of selective NKR antagonists and of the pan-NKR antagonist ZD6021.

Compound	Human	Dog	Gerbil	Rat
NK₁R				
ZD6021	8.6 ± 0.4	9.5 ± 0.2	9.0 ± 0.2	<6
RP67580	7.1 ± 0.4	7.1 ± 0.6	6.5 ± 0.2	7.3 ± 0.4
CP99,994	8.7 ± 0.2	9.8 ± 0.3	8.9 ± 0.3	5.9 ± 0.2
Aprepitant	8.7 ± 0.2	9.2 ± 0.1	8.8 ± 0.2	7.3 ± 0.1
NK₂R				
ZD6021	8.3 ± 0.4	8.4 ± 0.2	8.4 ± 0.3	8.1 ± 0.1
Saredutant	9.1	9.4 ± 0.1	9.3 ± 0.2	9.4 ± 0.1
NK₃R				
ZD6021	7.9 ± 0.3	7.8 ± 0.1	7.9 ± 0.1	6.7 ± 0.2
Talnetant	8.6 ± 0.3	8.4 ± 0.2	8.4 ± 0.1	7.4 ± 0.2
Osanetant	8.4 ± 0.5	8.2 ± 0.2	8.0 ± 0.2	7.4 ± 0.3