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NEUROKININ (NK)1 RECEPTOR ANTAGONISTS: CORRELATION BETWEEN *IN VITRO* RECEPTOR INTERACTION AND *IN VIVO* EFFICACY

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Abbreviations: NK, Neurokinin; SP, Substance P; NKA, Neurokinin A; hNK_1R, human NK_1 receptor; GFT, Gerbil Foot Tap; CHO, Chinese Hamster Ovary

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ABSTRACT

We compared the neurokinin 1 receptor (NK_1R) antagonists aprepitant, CP99,994 and ZD6021 with respect to receptor interactions and duration of efficacy *in vivo*. In Ca^{2+} mobilization assays (FLIPR), antagonists were applied to human U373MG cells simultaneously with or 2.5 min before substance P (SP). In reversibility studies, antagonists were present for 30 min before washing and responses to SP were repeatedly measured afterwards. The compounds were administered i.p. to gerbils and the gerbil foot tap (GFT) response was monitored at various time points. The NK_1R receptor occupancy for aprepitant was determined in striatal regions. Levels of compound in brain and plasma were measured. Antagonists were equipotent at human NK_1R and acted competitively with SP. After pre-incubation, aprepitant and ZD6021 attenuated the maximal responses, while CP99,994 only shifted the SP concentration-response curve to the right. The inhibitory effect of CP99,994 was over within 30 min while for ZD6021, 50% inhibition still persisted after 60 min. Aprepitant produced maximal inhibition lasting at least 60 min. CP99,994 (3 μmol/kg) inhibited GFT by 100% 15 min after administration but the effect declined rapidly together with brain levels thereafter. The efficacy of ZD6021 (10 μmol/kg) lasted 4h and correlated well with brain levels. Aprepitant (3 μmol/kg) inhibited GFT and occupied striatal NK_1R by 100% for >48h despite brain levels of compound were below the limit of detection after 24h. Slow functional reversibility is associated with long-lasting *in vivo* efficacy of NK_1R antagonists while the efficacy of compounds with rapid reversibility is reflected by their pharmacokinetics.

Introduction

The tachykinins neurokinins substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) belong to the tachykinin peptide family (Severini et al., 2002). The tachykinin receptors are divided into three subtypes; NK_1R, NK_2R and NK_3R. The rank order of potency of the endogenous tachykinins are for NK_1R: SP ≥ NKA > NKB, for NK_2R: NKA > NKB > SP and for NK_3R: NKB > NKA > SP (for review see Pennefather et al., 2004). Hemokinin-1 (HK-1) and endokinins A and B (EKA and EKB) are relatively new mammalian members of the tachykinin family but appear to have similar receptor pharmacology as SP (Page, 2006). On the other hand, endokinins C and D have negligible affinity for known NK receptors (Page, 2006).

Preclinical research has implicated especially the NK_1R as being involved in several pathological disorders including emesis, asthma, psychiatric disorders, gastrointestinal disorders, pain, migraine, inflammation and urinary bladder disorders. This has led to the subsequent development of selective and potent NK_1R antagonists (for a recent review see Quartara and Altamura, 2006). However, so far only aprepitant has reached the market for treatment of chemotherapy-induced emesis.

To date, still little is known about the way antagonists interact with NK_1R and, especially, about the mechanisms that govern the duration of their effects *in vivo*. The *in vivo* efficacy of an antagonist and it´s duration of action can sometimes be difficult to predict based only on potency values obtained by *in vitro* assays (Copeland et al., 2006). To provide more information, two additional approaches have often been used in *in vitro* pharmacological studies. One consists in exposing antagonist-pretreated tissue or cells to fresh medium and monitoring the restoration of receptor responsiveness to an agonist. Such functional wash-out experiments, not only provide information about the functional dissociation rate of the antagonist-receptor complex,

but under appropriate conditions, also provide information about the likelihood of the liberated antagonist to undergo fast rebinding to receptors in the neighborhood of where they were released (Lullmann et al., 1988; Fierens et al., 1999a; Chu et al., 2004).

The second approach consists in monitoring antagonists for their potential to be insurmountable, i.e. for their capability to decrease the maximal response that can be elicited by a subsequently added agonist (Vauquelin et al., 2002a). Although this approach has been most often used in "organ bath" experiments with intact tissues, it can also be successfully applied in intact cell-based experiments (Vauquelin et al., 2002b). As an illustration of this approach, *in vitro* assays with NK_1R expressing cells pointed at a causual link between the insurmountable behaviour of the competitive NK_1R-selective antagonists SR140333 and aprepitant and their slow rate of dissociation from the receptor (Emonds-Alt et al., 1993; Hale et al., 1998). That slow dissociation may produce insurmountable inhibition can easily be explained by the fact that the antagonist fails to liberate all the receptor sites during the ensuing challenge with the agonist so that the measured response is sub-optimal. On the other hand, the surmountable behaviour of fast dissociating antagonists is likely to reflect a swift liberation of the receptors.

However, insurmountable antagonism can also be explained by non-competitive interactions. This latter mechanism has been been held responsible for the behaviour of the NK_1R-selective antagonist CP122,721 (McLean et al., 1996). These studies illustrate that still little is known about the way antagonists interact with NK_1R.

In the present study we compare three different NK receptor antagonists with respect to their functional interactions *in vitro* and how these interactions correlate to effect duration *in vivo*. The study has been performed in U373MG cells endogenously

expressing the human NK_1R (Eistetter et al., 1992). The experiments *in vitro* were designed to evaluate competitive and insurmountable NK_1R interactions and functional reversibility was tested after pre-treatment of antagonist. Gerbils represent a species with similar NK_1R pharmacology to man (Beresford et al., 1991, Engberg et al., 2007). We therefore investigated the pharmacokinetic/pharmacodynamic (PK/PD) relationship of the compounds *in vivo* using the gerbil foot tap (GFT) assay, which is a model reflecting central NK_1R activation (Bristow and Young, 1994). We also determined the degree of NK_1R occupancy for aprepitant in gerbil striatum using autoradiography in order to verify the prolonged effect *in vivo*.

Methods

Chemicals

The selective NK_1R antagonists CP99,994 (McLean et al., 1993) and aprepitant (Hale et al., 1998) and the pan-NK receptor antagonist ZD6021 (Bernstein et al., 2001) were synthesized at AstraZeneca.

Cells

Human glioblastoma astrocytoma (U373MG) cells endogenously expressing NK_1R were used (European collection of cultures 89081403, Sigma Aldrich, St Louis, MO, USA). The cells were cultured in a humidified incubator under 5% CO_2 in MEM with Earle´s medium and glutamax, 10% FBS, 1% NEAA and 1% MEM-sodium pyruvate. The cells were grown in T175 flasks and passaged when 70-80% confluency was achived for up to a maximal of 20 passages. Prior to each experiment, U373MG cells were plated in black-walled/clear-bottomed 96-well plates (Costar 3904) at 2.5 x 10^4

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cells per well and grown for approximately 24 h in normal growth media in a 37°C CO_2-incubator in order to achieve confluency.

Intracellular measurements of Ca^{2+}

U373MG cells, grown in 96-well plates, were loaded with the Ca^{2+} sensitive dye Fluo-4 (Teflabs 0152, Austin, TX, USA) at 4 µM in a loading media consisting of Nut Mix F12 (HAM) with glutamax I, 22 mM HEPES, 2.5 mM probenicid (Sigma, P-8761) and 0.04% pluronic F-127 (Sigma, P-2443) and kept dark for 30 min in a 37°C CO_2-incubator. The cells were then washed three times in assay buffer which consisted of Hanks´ balanced salt solution containing 20 mM HEPES, 2.5 mM probenicid and 0.1% BSA, using a multi-channel pipette leaving them in 150 µl at the end of the last wash. Serial dilutions of test compound in assay buffer (final DMSO concentration kept below 1%) and/or agonist were automatically pipetted into each test well and the peak fluorescence intensity was recorded (λ_{ex} 488 nm and λ_{em} 540 nm) by the FLIPR CCD camera for approximately 2.5 min. The response was measured as the peak relative fluorescence after agonist addition. The potency of the antagonists used were determined using the same methodology but with CHO-K1 cells transfected with human NK1R (Engberg et al., 2007).

Co-incubation experiments

To test for competitive interactions, a co-incubation procedure was used by adding aprepitant, ZD6021 or CP99,994 (at final concentrations ranging from 40 - 640 nM) to the wells by the FLIPR automatic station simultaneously with increasing concentrations of SP.

Pre-incubation experiments

To test for insurmountable interactions, a pre-incubation protocol was used by adding aprepitant, ZD6021 or CP99,994 (at final concentrations ranging from 1 - 40 nM) to the wells by the FLIPR automatic station 2.5 min prior to addition of increasing concentrations of SP.

Reversibility of NK_1R antagonist effect

U373MG cells, seeded in 96-well plates, were loaded with 4 µM Fluo-4 (see above) together with 10 nM of aprepitant, ZD6021, CP99,994 or loading buffer (controls) and kept dark for 30 min in a 37°C CO_2-incubator. The plates were then washed 3 times in assay buffer (see above) leaving the cells in 150 µl assay buffer at the end of the last wash. The cells were then incubated for 1, 3, 10, 30 or 60 minutes at 37°C in a CO_2-incubator before a SP solution (final concentration 3 nM) was automatically pipetted.

Gerbil foot tap experiments

Male Mongolian gerbils (60 – 80 g) were purchased from Charles River (Sulzfeld, Germany). On arrival, they were housed in groups of ten in cages (height: 40 cm, width: 80 cm, length: 60 cm) containing an enriched environment including hay, plastic tubes, nesting material and sand. Food and water were available *ad libitium* and the cages were placed in temperature and humidity-controlled holding rooms. The animals were allowed at least 7 days to acclimatize to the housing conditions before experiments. All experiments were approved by the local animal ethical committee of Göteborg, Sweden.

Compounds and corresponding vehicles were administered under brief isoflurane (Forene®, Abbott Scandanavia AB, Solna, Sweden) anaesthesia. A dose of 3 μmol/kg aprepitant (dissolved in ethanol/solutol/saline 5/5/90) or 10 μmol/kg ZD6021 (dissolved in 28% cyclodextrin) or 3 μmol/kg of CP99,994 (dissolved in saline) or corresponding vehicle was administered i.p. at various time points before the experiment. At the indicated time point after compound administration, the animals were anaesthetised (isoflurane), and a small incision was made in the skin over bregma. Ten pmol of acetyl-[Arg^6,Sar^9,$Met(O_2)^{11}$]-SP6-11 (ASMSP), a selective agonist for NK1 receptors, was administered i.c.v. in a volume of 5 μl using a Hamilton syringe with a needle 4.5 mm long. The wound was clamped shut and the animal was allowed to recover in a small plastic cage. The cage was placed on a piece of plastic tubing filled with water and connected to a computer via a pressure transducer. The number of taps produced by the animal were recorded for 6 min using customized computer software (PharmLab on-line 4.0, AstraZeneca, Mölndal, Sweden). The average number of taps per minute during the middle 5 min was calculated (thus the first and last 30 s were excluded). Ten pmol ASMSP typically evoked an average of 100 taps per minute. Antagonist efficacy was expressed as % inhibition in comparison to corresponding vehicle. After the experiment, the animals were sacrificed under anaesthesia by exsanguination of the heart. Half of the brain together with plasma were removed in order to determine levels of compound. In aprepitant experiments, the other half of brain was used for autoradiography (see below).

Determination of compound concentrations in brain and plasma

The collected brains were thawed and 3 ml of water per gram brain tissue was added. The brain was homogenised by ultrasonication and the brain homogenate and plasma samples were stored at -20°C until analysis. Brain homogenate and plasma samples (50 µl) were protein precipitated by addition of 150 µl acetonitrile containing 0.2 % formic acid and internal standard. After vortexing, the samples were centrifuged for 20 min at 2900 g and 4°C. The supernatant (75 µl) was diluted with 75 µl of 0.2 % formic acid in water. Brain homogenate and plasma samples were analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). An Agilent 1100 LC pump (Agilent Technologies, Waldbronn, Germany) was used with gradient elution using a flow rate at 0.6 ml/min. The mobile phase consisted of (A) 2% acetonitrile and 0.2% formic acid in water and (B) 0.2% formic acid in acetonitrile. Separation was performed on a 30 x 2.1 mm C18 HyPURITY column with 5 µm particle size (Thermo Electron Corporation, Waltham, MA, USA) using a linear gradient of 5 - 90% B for 2 min, held at 90% for 1 min and returned to initial conditions in one step. The front was diverted to waste by using a 6-port valve (VICI AG, Schenkon, Switzerland) and after 0.5 min the effluent entered the MS without splitting. Sample storage and injection was performed with a CTC HTS Pal autosampler (CTC Analytics, Zwingen, Switzerland). Detection was performed with positive electrospray ionization mode by multiple reaction monitoring (MRM) using a Micromass Quattro LC triple quadropole (Waters, Manchester, UK). Instrument control, data acquisition and data evaluation were performed using Masslynx 4.0..

Autoradiography and binding experiments

Following a single dose of aprepitant (3 µmol/kg i.p., see GFT experiments above), the animals were sacrificed after various time points (0.5, 1, 2, 4, 8, 24, 48 and 72 h).

The brains were rapidly removed and frozen on dry ice and stored in -80°C until further use. Sagittal frozen sections (16 µm) were sectioned in a cryostat at -15°C and thaw-mounted on SuperFrost®Plus section slides (Menzel Gmbh & Co KG, Braunschweig, Germany), and stored at -80°C until use. Tissue sections were pre-incubated at room temperature for 5 min in 50 mM Tris-HCl containing 0.3% bovine serum albumin (BSA). Sections were then incubated at room temperature in 50 mM Tris-HCl (pH 7.4) containing 0.1% BSA, 40 µg/ml bacitracin, 3 mM $MnCl_2$ and Complete™ EDTA-free protease inhibitor cocktail tablets (Roche, Mannheim, Germany) for 60 min in the presence of 4 nM [^{3}H]-Sar,Met(O_2)-Substance P. CP99,994 (10 µM) was used to assess non-specific binding. The sections were subsequently washed in 50 mM ice-cold Tris-HCl (pH 7.4) for 2 x 5 min, briefly dipped in ice-cold distilled water and then dried. The sections were placed in hyper-cassettes and exposed 4 days to imaging plates with [^{3}H]-microscales (Amersham) as standard. Imaging plates were scanned using a BAS-5000 Bio-Imaging Analyzer (Fuji Photo Film, Tokyo, Japan) and quantified using an image analysis software system (AIDA 4.10, Raytest, Straubenhardt, Germany) in order to measure optical densities. Ligand binding in the striatum was monitored since the NK_1R is most abundant in this region in gerbil (Griffante et al., 2006), monkey (Bergström et al., 2000) and man (Hargreaves, 2002). Specific binding in the presence of [^{3}H]-Sar,Met(O_2)-Substance P was set to 100% while non-specific binding in the presence of 10 µM CP99,994 was set to 0%.

Data analysis

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Curve fitting, IC_{50} and EC_{50} estimations were carried out using a four-parameter logistic model in XL*fit* for Microsoft Excel. Data are expressed as mean values ± S.E.M..

Results

Effect of NK receptor antagonists on SP concentration-response curves.

In vitro antagonist interactions were montiored using U373MG cells which endogenously express the hNK_1R. In co-incubation experiments, all three antagonists produced a rightward shift of the SP concentration-response curve (Figs 1A-C). Maximal responses to SP and Hill slopes remained the same, indicating a competitive interaction. The potency (pK_B-values) for aprepitant, ZD6021 and CP99,994 in CHO-K1 cells were 8.7 ± 0.2, 8.7 ± 0.2 and 8.6 ± 0.4, respectively.

In pre-incubation experiments (antagonist added 2.5 min before SP), aprepitant suppressed the maximal response to SP in a concentration-dependent manner (Figs. 1D-F). Pre-incubation with 1 nM aprepitant attenuated the maximal response to SP, while 10 nM virtually abolished the response (Fig. 1D). ZD6021 also suppressed the maximal response to SP in a concentration-dependent manner in pre-incubation experiments (Fig.1E). When compared to the efffect of aprepitant, the suppression by ZD6021 was less complete. In contrast, pre-incubation with all concentrations of CP99,994 produced a rightward shift of the SP concentration-response curve with maintained maximal SP-evoked responses (Fig. 1F)

Reversibility of NK receptor antagonist inhibition

Pre-incubation with 10 nM aprepitant produced long-lasting inhibition of SP-evoked responses (Fig. 2). The response to SP was not restored following 60 min washout of aprepitant. ZD6021 (10 nM) also produced time-dependent inhibition of SP-evoked responses resulting in ~60% inhibition after 1 h. On the other hand, inhibition produced by pre-incubation of 10 nM CP99,994 was completely reversed within less

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than 30 min. The washout procedure *per se* did not affect the ability of SP to evoke increases in intracellular Ca^{2+}.

Effect of NK receptor antagonists in vivo

Aprepitant (3 μmol/kg i.p.) produced a long-lasting complete inhibition of the GFT response (Fig 3A). Maximal inhibition was attained after 2 h and brain levels peaked at this time point reaching 450 nmol/kg. After 4 h, aprepitant levels in the brain started to decline, however a full inhibitory response (100%) was maintained. At 48 h, levels of aprepitant in the brain were below the limit of quantification (10 nmol/kg) but a prominent inhibitory effect (80 ± 13%) was still present. The time-dependent inhibitory response elicited by aprepitant correlated extremely well with the degree of NK_1R brain occupancy in autoradiography studies (Fig. 4). After 72h, both the ASMSP-evoked GFT response and the NK_1R occupancy by radioligand was restored (Fig.4).

ZD6021 (10 μmol/kg, i.p.) also inhibited GFT with maximal effects (69 ± 11%) appearing after 1 h (Fig 3B). The levels of ZD6021 peaked already at 30 min reaching 123 ± 14 nmol/kg. The inhibitory effect and brain levels of ZD6021 slowly decreased after 1 h. At 8 h, levels of ZD6021 were below the level of quantification (10 nmol/kg) and the inhibitory effect had subsided.

Treatment with CP99,994 (3 μmol/kg, i.p.) resulted in complete inhibiton of GFT 15 min after treatment (Fig 3C). The inhibitory effects were relatively short-lasting and reflected brain levels of compound which declined rapidly after 15 min.

A summary of maximal compound levels detected in plasma and brain is shown in Table 1.

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Discussion

The present study compares the *in vitro* NK_1R interaction properties of the nonpeptide antagonists aprepitant, ZD6021 and CP99,994 with time-wise changes in blockade of NK_1R function *in vivo*. Assays on intact U373MG cells which endogenously express human NK_1R showed that all compounds are competitive antagonists with similar potency, but that there is a marked difference in the duration of receptor blockade: i.e. aprepitant >> ZD6021 > CP99,994. The *in vitro* interaction properties of aprepitant correlate well with long-lasting functional GFT inhibition and *in vivo* NK_1R occupancy in the gerbil CNS.

Earlier human and rabbit pulmonary artery relaxation and guinea pig ileum contraction studies already revealed that NK_1R receptor antagonists like SR140333, CP122,721 and MEN 11149 decrease the maximal response to substance P or related agonists (Emonds-Alt et al., 1993; Croci et al., 1995; Cirillo et al., 1998; Pedersen et al., 2000). As usual for such organ-bath experiments (Leff and Martin, 1986), the tissues were pre-incubated with the antagonist before their challenge with agonist. Antagonists which inhibit the maximal response under such conditions are referred to as insurmountable (Gaddum, 1955; Vauquelin et al., 2002a,b). This type of antagonism can also be demonstrated to take place in cell lines provided that they are exposed to the antagonists before their challenge with an agonist (Fierens et al., 1999a). In the present study on human glioblastoma astrocytoma (U373MG) cells endogenously expressing NK_1R, aprepitant produced a complete and ZD6021 a nearly complete decline in the substance P-mediated cytosolic Ca^{2+} transients. In contrast, CP99,994

acted surmountably, i.e. it only produced a rightward shift of the substance P concentration-response curve.

Several models have been proposed to explain the operative mechanism of insurmountable antagonism. The most cited ones refer to non-competitive interactions, including functional inhibition (i.e. blockade of an essential step in the agonist-induced chain of cellular events) and binding to an allosteric site at the receptor, as well as to competitive interactions (i.e. binding of the antagonist and agonist to at least partially overlapping sites at the receptor) but associated with slow antagonist dissociation (Vauquelin et al., 2002a,b). When the receptors are allowed to pre-equilibrate with the antagonist, these scenarios all lead to a reduction in receptor activity and are therefore difficult to resolve (Bond et al., 1989). In contrast, co-incubation experiments allow a clear-cut discrimination since, in that case, competitive antagonists no longer decrease the maximal agonist-evoked response while noncompetitive antagonists still do (Fierens et al., 1999b). In such co-incubation experiments, aprepitant, ZD6021 and CP99,994 only produced parallell rightward shifts of the substance P concentration-response curves. This clearly points at the competitive nature of these antagonists and, hence, at a potential link between their degree of insurmountability and their dissociation rate from the receptor.

Antagonist dissociation from the NK_1R was monitored by functional "wash-out" experiments involving preincubation of the U373MG cells with saturating concentrations of aprepitant, ZD6021 and CP99,994, washing and exposure to fresh medium for the indicated periods of time before measuring the maximal substance P-mediated calcium transients. In this experimental paradigm, the rate by which the response is restored depends on the dissociation rate of the pre-formed antagonist-receptor complexes (Vanderheyden et al., 2000). Agonists are well known to promote

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the internalization of NK_1 receptors (and of GPCRs in general) via endocytotic processes. Among the several theories that have been put forward to explain insurmountable antagonism, it was proposed by Liu et al (1992) that it could reflect the ability of such compounds to promote receptor internalization as well. This model was specifically proposed for AT_1 receptor antagonists. However, subsequent confocal microscopic examinations revealed that nonpeptide antagonists did not affect the sub cellular distribution of fluorescent AT_1 receptor-green fluorescent protein conjugates (Hein et al., 1997, Le et al., 2005). Similar studies also indicate that nonpeptide NK_1 receptor antagonists are unable to induce receptor internalization and even that they will prevent SP-induced NK_1 receptor endocytosis and stress-induced NK_1 receptor internalization in the basolateral amygdala (Southwell et al., 1996; Jenkinson et al., 1999, Smith et al., 1999). Accordingly, presently available experimental evidence does not support the potential link between insurmountable antagonism and receptor internalization as proposed by Liu et al, (1992). In agreement with the surmountable behavior of CP99,994, the response was rapidly restored to the control level (i.e. the level in non-pretreated cells) for the CP99,994-pretreated cells. The restoration was appreciably slower (reaching about 40% of the control level after 60 min) for ZD6021-pretreated cells and even no restoration could be demonstrated within 60 min for the aprepitant-pretreated cells. These findings may explain the insurmountable behaviour of aprepitant and ZD6021 in the pre-incubation experiments. Indeed, these antagonists should have been unable to liberate a substantial part of the NK_1R during their subsequent challenge with substance P so that the maximally attainable response should be less than in the control situation, i.e. when all receptors are free at the moment of their challenge with agonist (Paton and Rang, 1966; Paton and Waud, 1967).

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Interestingly, slow dissociation has previously also been observed for other insurmountable NK_1R antagonists in organ bath wash-out experiments. The contractile response of SR 140333- pretreated guinea pig ileum to NK_1R stimulation took more than an hour to recover half-maximally (Emonds-Alt et al., 1993). Even slower recoveries of the response were recorded with FK888- and MEN 111149- pretreated guinea pig ilea (Cirillo et al., 1998). As the slow dissociation of those antagonists offers a sufficient explanation for their insurmountable behaviour, there is no strict necessity to invoke non-competitive interactions.

Whereas aprepitant produced a full decline of the maximal response in pre-incubation experiments, increasing the ZD6021 concentration first decreased the maximal response to a limit and then only produced rightward shifts of the substance P concentration-response curves. In the case of angiotensin AT_1 receptors, such partial insurmountability was also observed for antagonists like irebesartan, valsartan annd EXP3174 (Fierens et al., 1999b; Verheijen et al., 2002). To explain this behaviour, it was proposed that the antagonist- receptor complexes are able to adopt two distinct but interconverting states: a fast reversible state (for the surmountable inhibition), and a slow reversible state (for insurmountable inhibition) (Fierens et al., 1999b; Vauquelin et al., 2001). Although still speculative at the present level of investigation, such a model could also provide a simple explanation for the partial unsurmountable behavior of ZD6021.

The very slow dissociation of the aprepitant-NK_1R complexes in the present intact cell-based experiments coincides with its long-lasting *in vivo* occupancy of central NK_1R and its inhibitory effects in the GFT assay. In this respect, slow receptor dissociation has been proposed to contribute to the long-lasting clinical action of antagonists for angiotensin AT_1- (Wienen et al.,1993; Aiyar et al., 1995; De Arriba et

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al., 1996; Unger 1999), histamine H_1- (Anthes et al., 2002), nicotinic- (El Bizri and Clarke, 1994), adrenergic α_{2A}- (Kukkonen et al., 1997), serotonergic 5-HT_3- (Blower, 2003) and muscarinic M_3 receptors (Swinney, 2004). In this respect, recent simulation studies (Vauquelin and Van Liefde, 2006) reveal that, compared to a fast dissociating antagonist, prolonged *in vivo* receptor occupancy should take place when the antagonist-receptor complexes dissociate much slower than the antagonist gets eliminated. This implies that the duration of *in vivo* receptor protection by antagonists should not only depend on their rate of elimination via excretion and/or metabolism but also on the rate at which they dissociate from their receptor (Unger, 1999, Swinney, 2004). In line with this view, we show here that the sustained GFT-inhibiting efficacy of aprepitant reflect its *in vivo* NK_1R occupancy in the CNS rather than compound levels of at the site of action. Long-lasting effects of aprepitant in GFT have been reported previously (Hale et al., 1998, Duffy et al., 2002) and the excellent correlation between the sustained GFT inhibition and central NK_1R occupancy in the present study is also consistent with others (Duffy et al., 2002). The present study however extends these findings by demonstrating that prominent GFT-inhibiting efficacy of aprepitant persisted even when its CNS levels were below the limit of detection. This contrasts with the early phases of the treatment, where NK_1R occupancy and inhibition of GFT by aprepitant closely followed it´s CNS levels until maximal inhibition was attained after about 2 h.

Elegant studies using positron emission topography (PET) have been performed with aprepitant in man (see Keller et al., 2006 for a summary). Interestingly, the plasma levels required in man for 95% occupancy of central NK_1R were approximately 1 μg/ml (approximately equilvalent to 2 μmol/l). In the current study, plasma levels peaked at 0.77 μmol/l suggesting that the dose used in gerbils is similar to clinically

relevant doses in man although potential species differences in protein binding and brain/plasma ratios need to be taken into account.

Compounds metabolized to pharmacologically active metabolites are also likely to prolong effect duration *in vivo*. In ferrets, administration of aprepitant results in formation of metabolites with affinity for NK_1R (Huskey et al., 2003). However, the level of metabolites detected in ferret brain were much (> 4-fold) lower than the parent compound aprepitant. In addition, the metabolites had weaker affinity for the NK_1R (4 - 100-fold) suggesting that active metabolites do not play a role in mediating the pharmacological effects of aprepitant *in vivo*. To our knowledge, active metabolites of CP99,994 or ZD6021 have not been reported.

The pan NKR antagonist ZD6021 has been described to act as a competitive, surmountable antagonist at NK_1R and NK_2R in rabbit pulmonary arteries while having non-competitive interactions at NK_3R in guinea pig ileum (Rumsey et al 2001). These findings clearly differ with the partially insurmountable effect of ZD6021 in the current study on human NK_1R. This discrepancy could result from many causes, including species-related differences in receptor behavior as well as the much shorter challenge of the receptors with agonist before measuring the response in the present study. Also in contrast with partially insurmountable behaviour and the relatively slow reversibility of ZD6021 antagonism in the present *in vitro* wash-out experiments, the inhibitory effects of ZD6021 on GFT corresponded well with the *in vivo* CNS levels of this antagonist. This could be related to an unfavourable ratio between the half-life of the ZD6021-NK_1R complexes (about 1 h in the *in vitro* wash-out experiments) and the half-life of the compound in the CNS (about 3 h). Indeed, simulation studies (Vauquelin and Van Liefde, 2006) reveal that, even for slow dissociating antagonists, the *in vivo* receptor occupancy is mainly dictated by their rate of elimination if the

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half-life of the antagonist-receptor complexes is shorter. As the surmountable antagonist CP99,994 dissociates even faster than ZD6021, it is thus no surprise that its inhibitory effect on GFT closely followed its *in vivo* CNS levels. Both reached a peak after 15 min and rapidly declined afterwards.

Despite possessing similar potency at human NK_1R *in vitro*, the brain levels required for efficacy in GFT differed somewhat between antagonists. This is not due to species-related differences in NK_1R pharmacology since the pKb values for the antagonists at cloned gerbil NK_1R were 8.8 for aprepitant (unpublished observations) and 8.9 and 9.0 for ZD6021 and CP99,994 respectively (Engberg et al., 2007). These values correlate well when investigating antagonist potency at human NK_1R (8.7, 8.7 and 8.6 for aprepitant, ZD6021 and CP99,994 respectively). By contrast, differences in efficacy in GFT in relation to brain levels may be explained by compound-dependent differences in protein binding, resulting in different levels of free antagonist in the CNS.

In conclusion, the present results comply with earlier simulation studies (Vauquelin and Van Liefde, 2006) by showing that the antagonist aprepitant exhibits very slow NK_1R receptor dissociation *in vitro* and, likewise, produces long-lasting *in vivo* receptor blockade that cannot be explained by the time-wise decline of its free concentration. On the other hand, in compliance with their faster receptor dissociation *in vitro*, the *in vivo* effect duration of ZD6021 and CP99,994 is rather dictated by the pharmacokinetics of the compounds. The present findings also lend support to recent considerations (Copeland et al., 2006) about potential advantages of long receptor occupancy by a drug in terms of its pharmacological effect duration and the underlying need to allocate more attention to kinetic approaches in *in vitro* drug discovery studies.

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Legends for figures

Figure 1. Representative curves demonstrating the effect of aprepitant (**A**,**D**), ZD6021 (**B**,**E**) and CP99,994 (**C**,**F**) on SP-induced mobilization of intracellular Ca^{2+}. (**A-C**): the compounds were co-incubated with the agonist, substance P, at the indicated concentrations. (**D-F**): the compounds were added to the cells (pre-incubated) 2.5 min before the challenge with substance P.

Figure 2. Effect of aprepitant, ZD6021 and CP99,994 after washout on SP-induced mobilization of intracellular Ca^{2+}. Antagonists (10 nM) were incubated with the cells for 30 min. After washing, cells were incubated in fresh medium and 3 nM substance P was applied 1, 3, 10, 30 or 60 min later. Mean values ± S.E.M., n = 3.

Figure 3. Effect of (**A**): aprepitant, 3 μmol/kg i.p., (**B**): ZD6021, 10 μmol/kg i.p. and (**C**) CP99,994, 3 μmol/kg i.p., on GFT (closed circles, left y-axis) after various time points of administration. The corresponding levels of compound detected in brain homogenates are depicted on the right y-axis with open circles. Mean values ± S.E.M., n = 3-5.

Figure 4. (**A**): Comparison of 3 μmol/kg aprepitant i.p. on ASMSP-evoked GFT (closed circles) and corresponding NK_1R occupancy (open circles) at various time points after administration. Mean values ± S.E.M, n = 3-5. (**B**): Representative autoradiography experiment showing the location and degree of radioligand (4 nM [^{3}H]-Sar,Met(O_2)-Substance P) binding to NK_1R in gerbil striatum. (**TB**): total

radioligand binding, (**24h, 48h and 72h**): radioligand binding in tissues collected from gerbils treated with 3 μmol/kg aprepitant i.p., 24, 48 and 72h respectively. Specific binding was > 95%.

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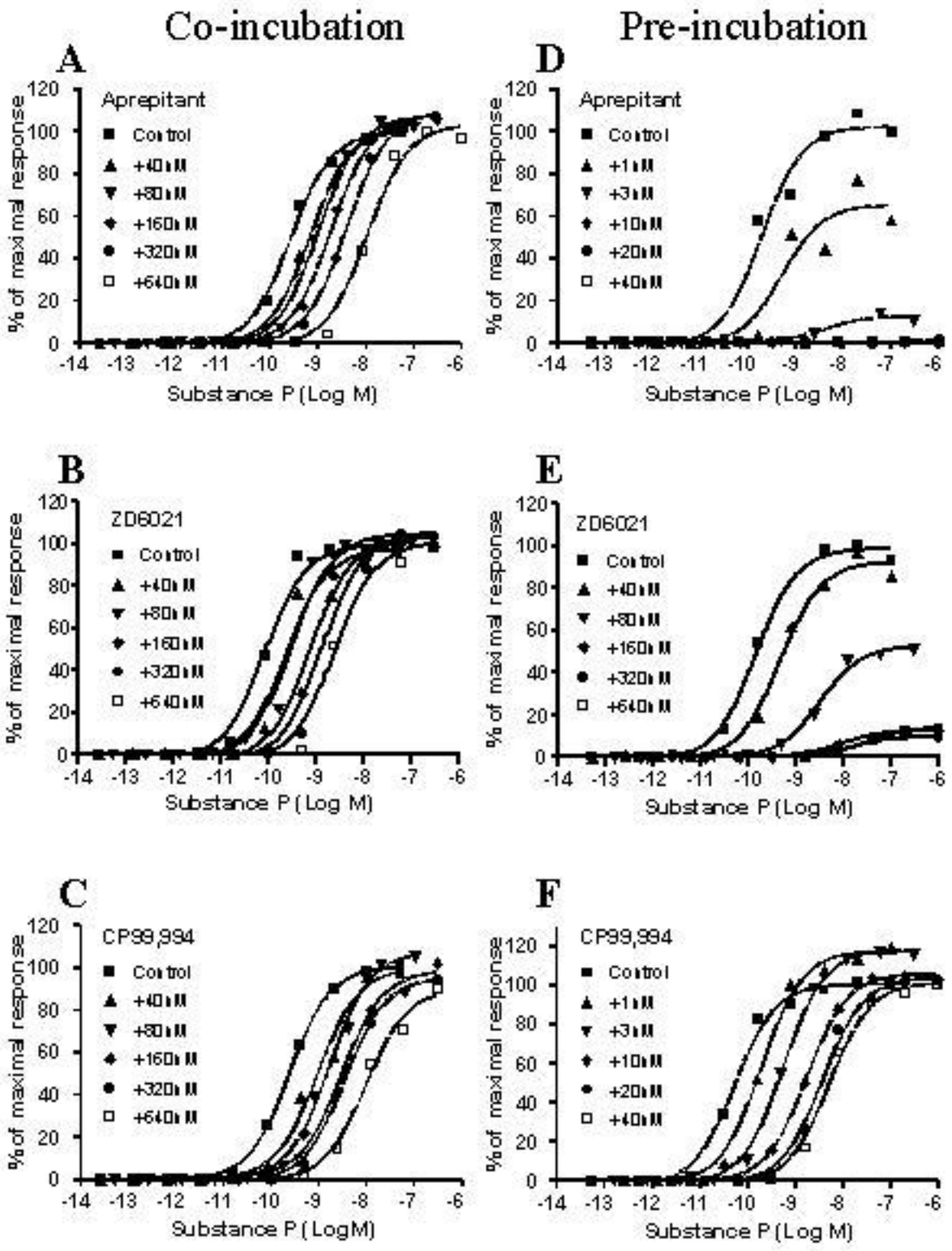
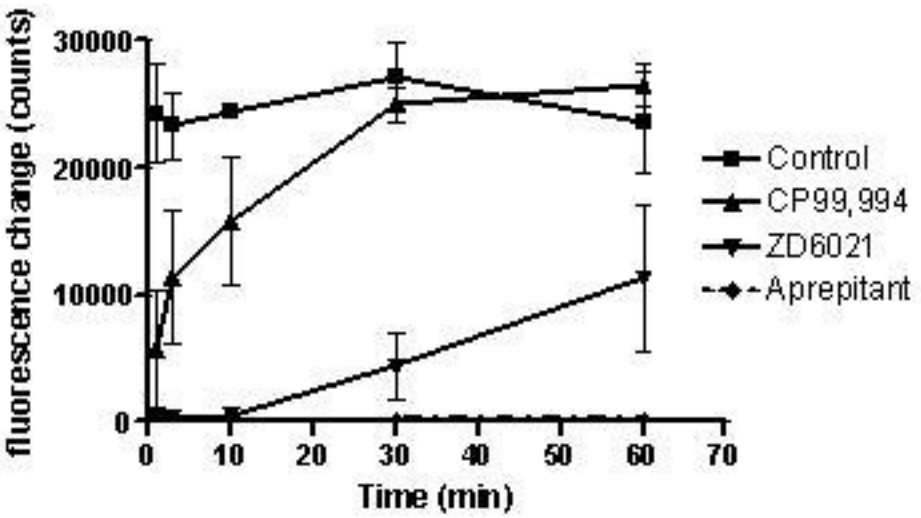
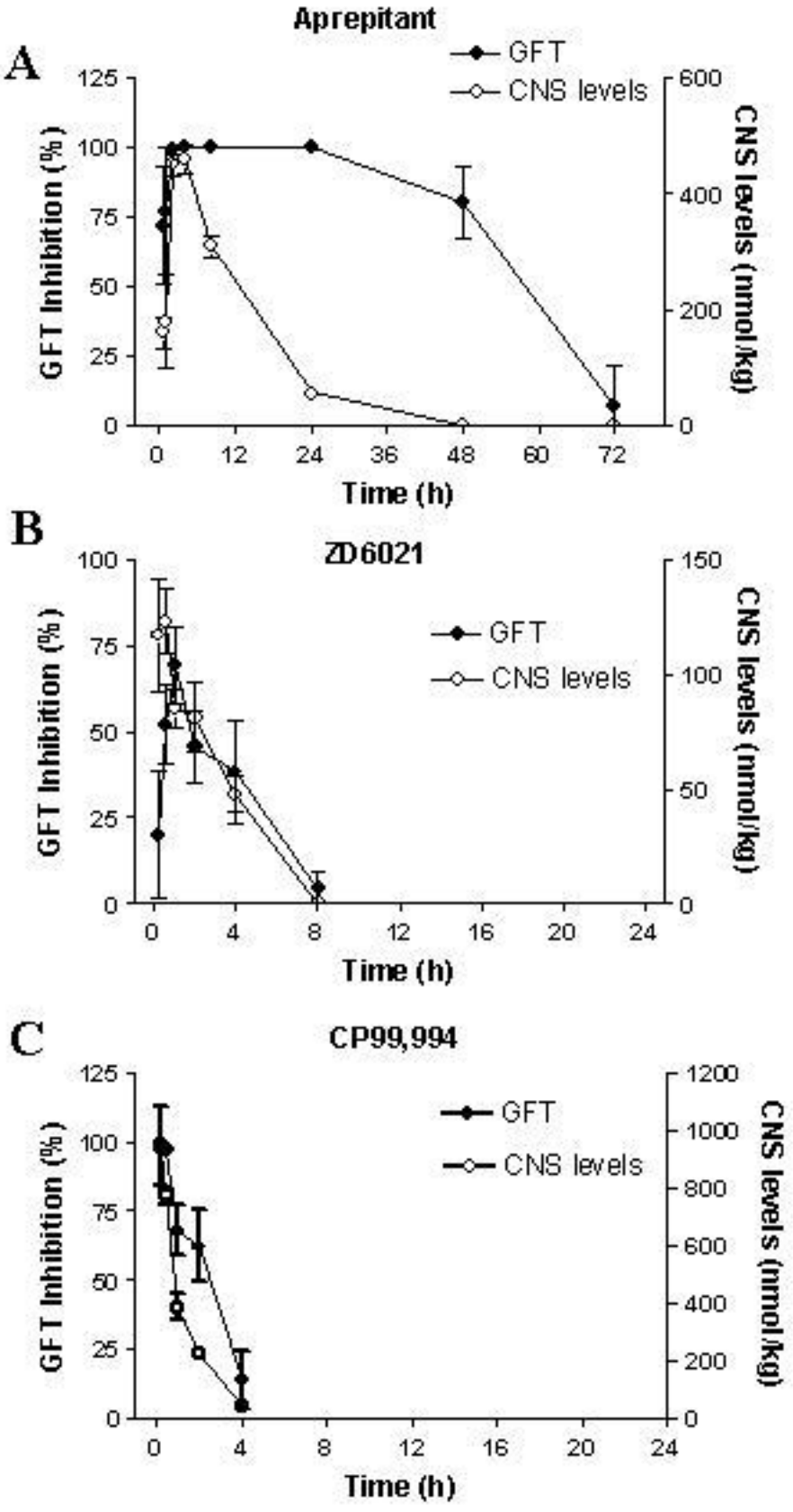
Table 1. Plasma and brain levels reached in gerbils after administration (i.p.) of NK_1R antagonists.

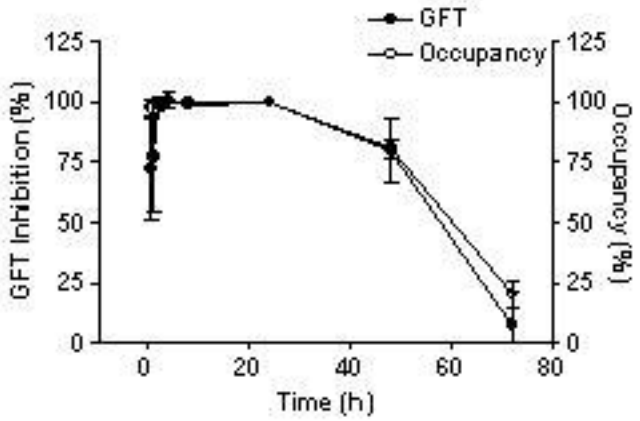
Compound	**Tmax plasma** (min)	**Cmax plasma** (nmol/l)	**Tmax brain** (min)	**Cmax brain** (nmol/kg)
Aprepitant	120	772 ± 27	240	460 ±26
ZD6021	30	1174 ± 240	30	123 ±14
CP99,994	15	35 ± 6	15	942 ±135


Co-incubation
Pre-incubation
A
D
B
E
C
F
Aprepitant
ZD6021
CP99,994
Control
+40nM
+80nM
+160nM
+320nM
+640nM
+1nM
+3nM
+10nM
+20nM
+40nM
% of maximal response
Substance P (Log M)
Substance P (LogM)

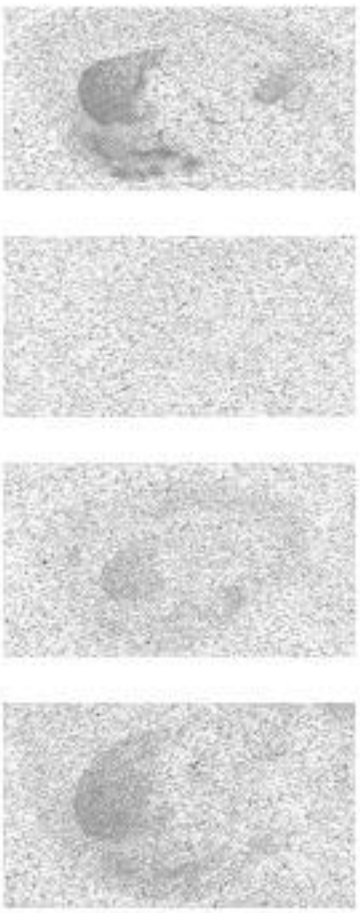

fluorescence change (counts)
30000
20000
10000
0
0
10
20
30
40
50
60
70
Time (min)
Control
CP99,994
ZD6021
Aprepitant


A
Aprepitant
GFT
CNS levels
GFT Inhibition (%)
CNS levels (nmol/kg)
Time (h)
B
ZD6021
GFT
CNS levels
GFT Inhibition (%)
CNS levels (nmol/kg)
Time (h)
C
CP99,994
GFT
CNS levels
GFT Inhibition (%)
CNS levels (nmol/kg)
Time (h)

A


GFT
Occupancy
GFT Inhibition (%)
Occupancy (%)
125
100
75
50
25
0
0
20
40
60
80
Time (h)

B


TB
24h
48h
72h