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Discovery and Biological Characterization of (2R,4S)-1′-Acetyl-N-{(1R)-1-[3,5-bis(trifluoromethyl)phenyl]ethyl}-2-(4-fluoro-2-methylphenyl)-N-methyl-4,4′-bipiperidine-1-carboxamide as a New Potent and Selective Neurokinin 1 (NK_1) Receptor Antagonist Clinical Candidate

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A large body of compelling preclinical evidence supports the clinical use of neurokinin (NK) receptor antagonists in a plethora of CNS and non-CNS therapeutic areas. The significant investment made in this area over the past 2 decades culminated with the observation that NK_1 receptor antagonists elicited clinical efficacy in major depression disorders. In addition, aprepitant (Merck) was launched as a new drug able to prevent chemotherapy-induced nausea and vomiting (CINV). After the discovery by GlaxoSmithKline of vestipitant, a wide drug discovery program was launched aimed at identifying additional clinical candidates. New compounds were designed to maximize affinity at the NK_1 receptor binding site while retaining suitable physicochemical characteristics to ensure excellent pharmacokinetic and pharmacodynamic properties in vivo. Herein we describe the discovery process of a new NK_1 receptor antagonist (casopitant) selected as clinical candidate and progressed into clinical studies to treat major depression disorders.

Introduction

Substance P (SP[a]), neurokinin (NK)A, and (NK)B[1] are all mammalian tachykinins whose biochemical effects are mediated by the G-protein-coupled receptors (GPCR) NK_1, NK_2, and NK_3. These neuropeptides, acting as both neurotransmitters and neuromodulators, are implicated in a variety of biological functions in the central nervous system (CNS), namely, pain transmission, inflammation, smooth muscle contraction, vasodilation, gland secretion, and activation of the immune system.[2−8] In particular, SP, the most abundant tachykinin in the CNS of mammals, along with its cognate NK_1 receptor, is present in brain regions such as the amygdala, septum, hippocampus, hypothalamus, and periaqueductal gray[9−11] cerebral areas involved in the regulation of affective behavior and the mediation of stress responses including anxiety and depression. In addition, SP was thought to play a critical role in emesis. As a consequence of this large therapeutic potential, several pharmaceutical companies have invested in this field since the beginning of the 1990s with the aim to identify selective and potent brain penetrant NK_1 receptor antagonists. Aprepitant (Merck), shown in Figure 1, has been approved and launched into the market for prevention of chemotherapy-induced nausea and vomiting (CINV). In our previous communications[12,13] we described the design process and the chemical exploration of a new *C*-phenylpiperazine template from which 2-(*S*)-(4-fluoro-2-methylphenyl)piperazine-1-carboxylic acid [1-(*R*)-(3,5-bis-trifluoromethylphenyl)ethyl]methylamide (vestipitant) **1** was derived.[14] Herein we report the evolution of that previous exploration aimed at identifying additional clinical candidates that exhibit appropriate druglike properties. The compounds synthesized were characterized, at recombinant human NK_1 receptors, in terms of in vitro receptor binding profile by displacement of [^{3}H]SP and in terms of functional activity, using fluorometric imaging plate reader (FLIPR) technology. As previously reported,[12] the latter aspect is fundamental for the selection of the most appropriate compounds to progress: a slowly dissociating NK_1 receptor antagonists showing nonsurmountable profile exerted a potent and longer lasting pharmacodynamic action than surmountable NK_1 receptor antagonists in the gerbil foot-tapping (GFT) model, which was chosen as the in vivo pharmacodynamic model of election to characterize new chemical entities (NCE) because of the similar in vitro pharmacology observed in human and gerbil NK_1 receptors. Conversely, NK_1 receptor antagonists were significantly less active in mice and rats than in man.[15] Moreover, over the past years,

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[a] Abbreviations: NK, neurokinin; SP, substance P; GPCR, G-protein-coupled receptor; CNS, central nervous system; SAD, social anxiety disorders; CINV, chemotherapy-induced nausea and vomiting; SAR, structure−activity relationship; CDI, carbonyldiimidazole; THF, tetrahydrofuran; CHO, Chinese hamster ovary; FLIPR, fluorescence imaging plate reader; GFT, gerbil foot tapping; CRC, concentration response curve; NCE, new chemical entity; Cl_i, intrinsic clearance.

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NK_1 receptor antagonists were found to be efficacious in preclinical in vivo models of affective behavior.[16-22] Hence, our compounds were characterized in the gerbil social interaction model, one of the most sensitive in vivo models to assess the anxiolytic-like profile of NK_1 receptor ligands.[23] Among the compounds synthesized, (2*R*,4*S*)-1′-acetyl-*N*-{(1*R*)-1-[3,5-bis(trifluoromethyl)phenyl]ethyl}-2-(4-fluoro-2-methylphenyl)-*N*-methyl-4,4′-bipiperidine-1-carboxamide methanesulfonate salt **16a** (casopitant) was found to be a potent, selective, orally active, and brain penetrant NK_1 receptor antagonist. Furthermore, as it had a favorable pharmacokinetic profile prediction in human, it was selected as a clinical candidate and progressed to clinical trials to treat major depression disorders.

Chemistry

The final compounds **9**−**20** were prepared as shown in Scheme 1. The racemate intermediate **3** was obtained by Comins-type reaction with 4-methoxypyridine which was treated with benzyl chloroformate and Grignard's reagent derived from commercially available 2-bromo-5-fluorotoluene. After the sequential reduction of the double bond with L-selectride and the following removal of the nitrogen protective group by catalytic hydrogenation, the racemic amine derivative **4** was purified by crystallization as camphorsulfonic acid salt. Then the pure enantiomer **5**, obtained by selective crystallization with L-mandelic acid, was coupled with *N*-methylbenzylamines **7a** and **7b**. The latter intermediate was synthesized by reductive amination of the ketone **6** followed by resolution of the racemic mixture by selective crystallization as L-malic acid salt. The next reaction was performed in the presence of triphosgene, to afford in good yield compounds **8a** and **8b**. Finally, the target compounds **9**−**20** were prepared from these common intermediates by reductive amination with $NaBH(OAc)_3$ in acetonitrile. In these reaction conditions a 4:1 mixture of the trans and cis diasteroisomers was obtained, which were easily purified by flash chromatography.

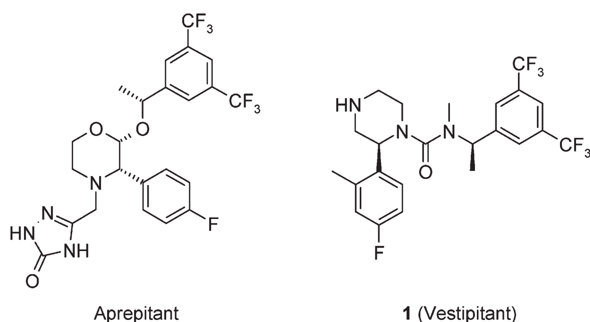
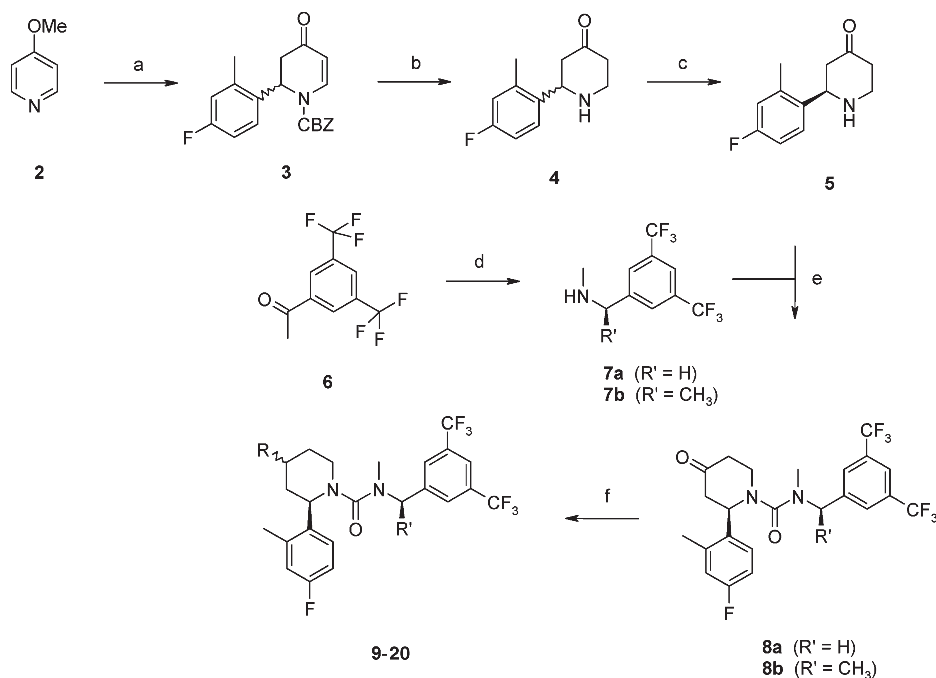


Figure 1. Chemical structure of aprepitant (Merck) and vestipitant (GSK).

Results and Discussion

As part of a large drug discovery effort aimed at the identification of new, druggable NK_1 receptor antagonists, the *C*-phenylpiperazine template, from which compound **1**, shown in Figure 1, was previously derived, was modified as shown in Figure 2. Keeping the urea moiety present within the right-end side chain constant (R = H or CH_3), we managed to modify the core of the template by moving the basic nitrogen out of the piperazine ring. This chemical modification enabled a wider exploration of the chemical space present in the left-end side region of the molecule with respect to the corresponding piperazines analogues. This was achieved by array synthesis

Scheme 1[a]



[a] (a) (i) 2-Bromo-5-fluorotoluene, Mg, THF, 60−70 °C; (ii) 4-methoxypyridine, benzyl chloroformate, THF, −20 °C, then Grignard's reagent, −20 °C, 1 h; (b) (i) tris(triphenylphosphine)rhodium(I) chloride, 2-propanol, H_2 (p = 5 atm), 60 °C, 5 h; (ii) Pd/C 5%, H_2 (p = 4 atm), 20 °C, 5 h; (iii) (*R*,*S*)-10-camphorsulfonic acid, toluene; (c) CH_2Cl_2, H_2O, 8% $NaHCO_3$ (aq); L-(+)-mandelic acid, 2-propanol, heptanes; (d) $MeNH_2$, EtOH, $NaBH_4$, 25 °C, 1.5 h; (e) (i) ethyl acetate, $NaHCO_3$ (aq. sat. soln), **5**; (ii) triphosgene, triethylamine, ethyl acetate, then **5**, 20 °C, 2 h; (f) R′RNH, CH_3CN, $NaBH(OAc)_3$, room temp, 24 h.

starting from ketone intermediates **8a** and **8b**, shown in Scheme 1, making use of an appropriate set of commercially available primary and secondary amines. Hence, following this synthetic strategy, a set of compounds was rapidly prepared and then characterized in terms in vitro affinity to the NK_1 receptor binding site by filtration binding assay on membranes derived from human (h)-NK_1-CHO cells, measuring the displacement of the radiolabeled natural agonist [^{3}H]SP, by increasing antagonist concentrations (1 pM to 10 μM). Then the most in vitro potent compounds were characterized in vivo, measuring the inhibitory effect (ID_{50}) of the characteristic animal's foot tapping evoked in male gerbils by intracerebroventricular (icv) infusion of the selective NK_1 receptor agonist GR73632.[12] Moreover, the gerbil social interaction model was used to assess the anxiolytic-like properties of the most interesting NK_1 receptor antagonist identified. From the set of in vitro affinity data obtained, summarized in Table 1, the following comments can be made: the replacement of the C-2 substituted piperazine core, present in compound **1**, by the corresponding 2-aryl-4-aminopiperidine resulted in the identification of an in vitro potent subfamily of NK_1 receptor antagonists. The high in vitro affinity observed was almost independent from the specific amine moiety introduced, accounting for the presence, within the NK_1 antagonists receptor binding site, of a sterically tolerant region able to accept bulky lipophilic and/or polar groups; cis diastereoisomers were consistently more potent than the corresponding trans diastereoisomers. No significant difference of the in vitro affinity was observed among secondary and tertiary amine derivatives synthesized (see pK_i values of compounds **17** and **10a**, pK_i = 9.70 and 9.62, respectively). As far as the type of substitution of the secondary amine is concerned, different kind of alkyl groups were tolerated (see compounds **17**, **18**, **19**, and **20**) with minimal impact on the value of the in vitro affinity at the NK_1 receptor. Finally, the in vitro affinity of the unsubtituted benzyl derivative (R′ = H) was slightly higher than that of the corresponding methyl substituted analogue (R′ = CH_3). However, the latter modification, as in the case of the pyrrolidine derivative, resulted in the obtaining of more potent NK_1 receptor antagonists (pK_i of 9.85 and 9.56 for **14a** and **13**, respectively), making the effect of this substitution subseries dependent.

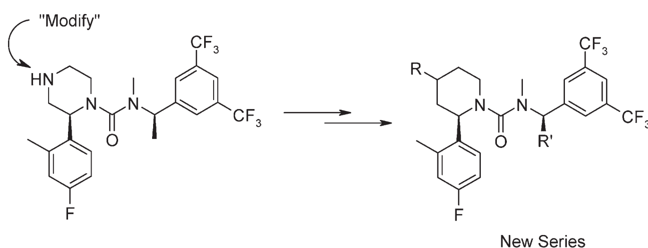


Figure 2. From vestipitant to the new chemical series.

The most in vitro active compounds, administered po 1 h before the icv administration of the NK_1 receptor agonist GR73632, exhibited excellent in vivo activity in the GFT model. In particular, the *N*-acetylpiperazine derivatives **15** and **16a**, when given po, emerged as the most potent molecules of the series (ID_{50} = 0.09 and 0.08 mg/kg, respectively). Notably, both compounds showed a long lasting inhibition of the foot tapping behavior, with the effect still being present

Table 1. In Vitro Binding Affinities (pK_i) at h-NK_1 Receptors and in Vivo Activity of NK_1 Receptor Antagonists in the Gerbil Foot Tapping Model (GFT)

compd	R	R′	stereochemistry[b]	pK_i[a]	*n*	ID_{50}, mg/kg (*t*)[c]
1 (vestipitant)				9.65	5	0.014
9	*N*,*N*-dimethylamine	H	cis	10.1	2	0.26
10a	*N*,*N*-dimethylamine	CH_3	cis	9.62	3	0.9
10b	*N*,*N*-dimethlylamine	CH_3	trans	9.03	2	NT[d]
11	azetidine	H	cis	10.09	2	0.13 (4 h)
12a	azetidine	CH_3	cis	9.75	2	NT[d]
12b	azetidine	CH_3	trans	9.19	2	NT[d]
13	pyrrolidine	H	cis	9.56	3	0.3
14a	pyrrolidine	CH_3	cis	9.85	2	0.92
14b	pyrrolidine	CH_3	trans	9.70	2	> 1 (4 h)
15	*N*-acetylpiperazine	H	cis	9.93	2	0.09
16a	*N*-acetylpiperazine	CH_3	cis	9.48	2	0.08
16b	*N*-acetylpiperazine	CH_3	trans	9.36	2	> 0.3
17	*N*-methylamine	CH_3	cis	9.70	2	NT[d]
18	*N*-cyclopropylamine	CH_3	cis	9.49	2	0.39 (4 h)
19	*N*-cyclobutylamine	CH_3	cis	9.77	2	NT[d]
20	2-methoxy-1-ethylamine	CH_3	cis	9.68	2	NT[d]

[a] pK_i values have been determined as described in Experimental Section, and *n* is the number of experiments performed in duplicate. [b] Relative stereochemistry of C-2 aryl group and the C-4 R substituent. [c] ID_{50} is the compound dose causing 50% inhibition of the tapping behavior elicited by the icv injection of GR73632, 3 pmol/5 μL. ID_{50} values are expressed in mg/kg after oral administration at *t* = 1 or 4 h (as shown in the table) before the test. [d] NT = not tested.

Table 2. ID_{50} for **16a** in the Gerbil Foot Tapping Model[a]

ID_{50}, mg/kg, sc (−1 h)	ID_{50}, mg/kg, po (−1 h)	ID_{50}, mg/kg, po (−4 h)	ID_{50}, mg/kg, po (−8 h)	ID_{50}, mg/kg, po (−24 h)
0.05 (0.02−0.06)	0.08 (0.04−0.60)	0.04 (0.04−0.40)	0.19 (0.09−0.50)	1.10 (0.74−1.96)

[a] Data are presented as ID_{50} values with 95% confidence limit. Seven animals per group were utilized in the test.

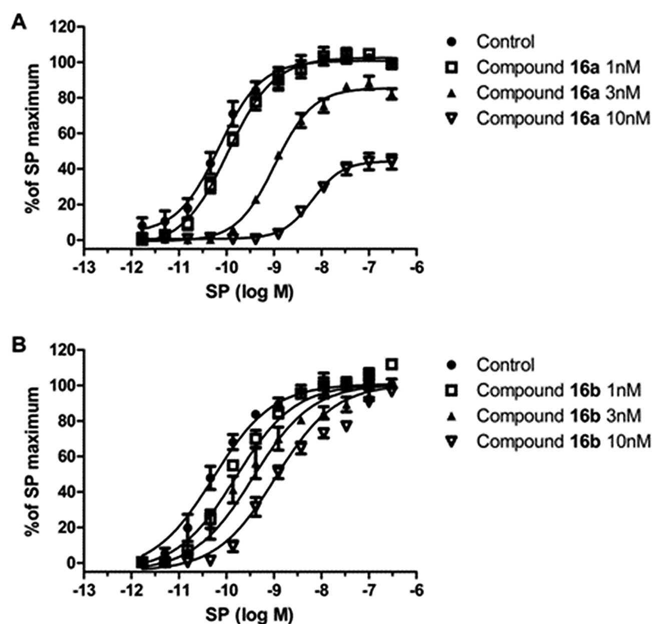


Figure 3. Antagonism of SP-induced increase of cytosolic calcium by **16a** and **16b** in h-NK1-CHO cells (each point is the average of two replicates each performed in duplicate).

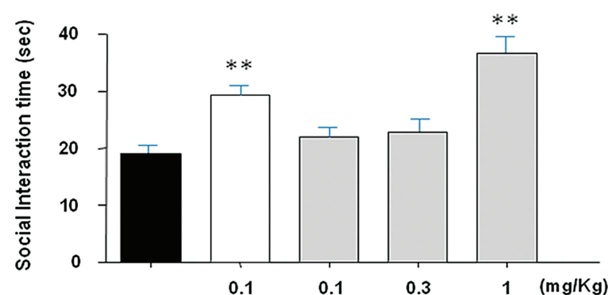


Figure 4. Increase of social interaction time (s) in gerbil produced by the ip administration of diazepam (white) and po administration of **16a** (gray) vs vehicle (black): (**) $p < 0.01$.

8 h after the oral administration of the compounds (ID_{50} = 0.08 and 0.19 mg/kg, respectively). A time course study was then carried out; remarkably, **16a** exhibited residual activity even after 24 h also (ID_{50} = 1.1 mg/kg), suggesting an appropriate exposure in the brain several hours after their systemic administration to the animals. As shown in Table 2, compound **16a** was also active after sc administration.

Surprisingly, the corresponding trans diastereoisomer **16b**, despite the similar in vitro affinity (pK_i = 9.36 vs 9.48 for compound **16a**), was found to be inactive up to 0.3 mg/kg in the GFT model (ID_{50} > 0.3 mg/kg) at both 1 and 4 h after its oral administration. This unexpected result might be explained by the different receptor mode of actions (MoA) observed for **16a** and **16b** as measured by the ability of these compounds to inhibit SP-induced release of cytosolic Ca^{2+} in h-NK_1-CHO cells using FLIPR (fluorometric imaging plate reader) technology, In particular, **16a** showed a nonsurmountable mode of action, whereas **16b**, as shown in Figure 3, conversely exhibited a surmountable mode of action. Apparent pA_2 values of 9.7 ± 0.1 (n = 3) for **16a** and 8.9 ± 0.1 (n = 2) for **16b** were calculated. Therefore, as previously highlighted for compound **1**, the nonsurmountable MoA was an associated feature of the receptor profile of the NK_1 receptor antagonist necessary to exert a robust and long lasting in vivo pharmacodynamic action. To assess the in vitro affinity at native NK_1 receptors, binding experiments were performed on homogenates derived from gerbil cerebral tissues, following the general protocol used for membranes obtained from h-NK_1-CHO cells as previously described.[24] Compound **16a** was tested in the 1 pM to 1 μM range of concentrations in the [^{3}H]SP filtration binding experiments on membranes prepared from gerbil brain cortices featuring a complete displacement of the specific binding of 0.8 nM [^{3}H]SP with a pK_i of 9.6 ± 0.2 (n = 3). In addition, [^{3}H]GR205171 (pK_i = 10.5 on h-NK_1) filtration binding experiments were carried out on homogenates prepared from human cortex. As in the previous case, **16a**, when tested from 1 pM to 1 μM, completely displaced [^{3}H]GR205171 present at 0.5 nM, with a pK_i of 9.9 (n = 2).

The receptor binding selectivity of compound **16a** was assessed at 10 μM in 70 different GPCRs, ion channels, and transporter systems. This study was performed by MDS Pharma Services according to their lead profiling screen (MDS Pharma Sevices 2001 catalogue, adding additional assays, namely, calcium channel type L, phenylalkylamine, NK_2, NK_3). The inhibition of specific binding was greater than 50% only for σ_1, sodium channel site 2, calcium channel type L, and dihydropyridine and benzothiazepine calcium channel type L. For these targets a complete displacement curve was carried out to provide K_i values. The calculated K_i values were 2.98 μM for σ_1, 4.22 μM for sodium channel site 2, 6.58 μM for dihydropyridine binding site calcium channel type L and for calcium channel type L, 8.78 μM for benzothiazepine binding site calcium channel type L. Hence, the receptogram screen confirmed **16a** as a very selective NK1 receptor antagonist. These in vitro and in vivo data highlighted two critical aspects for compound **16a**: (a) it possesses subnanomolar affinity and high selectivity for the NK_1 receptors without sign of pharmacological species difference between human and gerbil NK_1 receptors; (b) it is active in human native tissues and interacts at the same NK_1 receptor binding site as the chemically diverse NK_1 receptor antagonist GR205171.[25] Further to its promising biological profile, **16a** was selected as a NK_1 receptor antagonist satisfying the internal criteria for the progression of compounds and was characterized in vivo in the social interaction model in the gerbil. As shown in Figure 4, a 0.1 mg/kg dose of diazepam (the standard anxiolytic benzodiazepine) given ip, significantly increased gerbil social interaction (19 ± 1.2 s with vehicle, 29.3 ± 1.2 with diazepam, $p < 0.01$). No abnormal behaviors were recorded. Then compound **16a** was tested po at the doses of 0.1, 0.3, and 1.0 mg/kg. A dose dependent increase in time spent in active social interaction was observed. A significant increase was recorded at the highest dose tested

Table 3. Pharmacokinetic Parameters of Compound **16a** in Preclinical Species[a]

animal species[b]	Cl_p ((mL/min)/kg)	V_{ss} (l/kg)	half-life (h)	F (%)
mouse	120	4.0	1.1	21
rat	37	3.2	1.8	8
dog	10	1.9	4.5	34
cynomolgus monkey	26	3.0	2.1	8
marmoset	29	3.1	1.7	14

[a] PK parameters derived from plasma concentrations. [b] The PK profile was assessed in dog, cynomolgus monkey, and marmoset at 0.5 mg/kg iv and 1 mg/kg po at 1 mg/kg iv, at 2 mg/kg po in mouse, and at 1 mg/kg iv and po in rat.

(19.0 ± 1.2 s for vehicle to 36.6 ± 1.9 for **16a**, $p < 0.01$) with a maximal augmentation of 92% with respect to the vehicle treated animals. No effect on locomotor activity was observed. The data obtained indicate anxiolytic-like properties of **16a**, which parallels the profile observed for other NK_1 receptor antagonists in animal models of stress and anxiety.[23,26]

As far as the in vivo pharmacokinetic profile is concerned, **16a** was characterized in rodents (mouse and rat) and in dog and primates (marmoset and cynomolgus monkey). As shown in Table 3, this compound showed moderate to high plasma clearance greater than 50% of liver blood flow in all species and a moderate distribution volumes (V_d) again in all species tested; the lowest value observed was in dog (V_d = 1.9 L/kg). As a result of this characteristic, the observed half-life values were relatively short and the oral bioavailability was low in most species (8−14%), with the only exception being dog (34%). Subsequent in vitro and in vivo studies indicated that the poor oral bioavailability observed was most likely driven by an extensive first pass extraction; therefore, clearance was the main indicator to follow for optimizing oral bioavailability. On the basis of these results, the pharmacokinetics in human was not expected to be favorable. However, when the in vitro intrinsic clearance (Cl_i) studies were performed in human liver hepatocytes obtained from three different donors, Cl_i values of 0.49, 0.19, 0.07 mL/(min g), respectively, were observed, which therefore predicted a more favorable clinical scenario. The predicted human blood clearance along with allometric scaling suggested in fact that the clearance in human was potentially low, ranging from 140 to 553 mL/min with an elimination half-life in the range from 11 to 18 h, adequate enough for a once a day administration dose regime, a key requirement for the progression of the compound. The predicted low clearance in human, together with a good correlation between oral bioavailability and hepatic extraction ratios in preclinical species, indicated that oral bioavailability in human was expected to be higher than in animals (> 30%).

On the basis of this information, **16a** was progressed into preclinical development and, upon completion of the required toxicological package, to clinical studies. Notably, the pharmacokinetics generated subsequently during the first time in human phase were in good agreement with those predicted previously, with oral bioavailability and half-lives adequate for a once a day dose regime for clinical use.

Conclusion

An appropriate modification of the *C*-phenylpiperazine template enabled our medicinal chemistry team to expand the scope of the exploration of the series and to identify some in vitro potent and selective NK_1 receptor antagonist exhibiting remarkable druglike properties. In particular, the introduction of an *N*-acetylpiperazine moiety in the left-hand side region of the molecule resulted in the identification of compound **16a** as one of the most in vivo potent NK_1 receptor antagonist ever identified. In particular, this compound was found to be a potent and long lasting inhibitor of the foot tapping behavior induced in the gerbil following icv administration of the SP agonist GR73632. This finding together with the nonsurmountable receptor antagonism of **16a** suggests that this compound may possesses a similar NK_1 receptor kinetic profile as vestipitant, which showed a slow off-rate from the receptor complex.[12] Notably, it has been recently reported that slowly dissociating antagonists could exert substantially longer efficient receptor protection in vivo than surmountable antagonists,[27,28] thus making this attribute as an important feature to optimize the pharmacodynamic profile of GCPR antagonists. Moreover, the evaluation in the gerbil social interaction test revealed that **16a** also possesses an anxiolytic-like property, confirming the preclinical profile of NK_1 receptor antagonists on emotional behavior. Despite the suboptimal in vivo pharmacokinetics observed in different animal species, **16a** was progressed based upon the extrapolation of the in vitro clearance performed in human liver hepatocytes, predicting half-life and oral biovailability to be adequate for a once a day dose regime for clinical use, with the profile fully confirmed later in clinical studies.

Experimental Section

NMR spectra were recorded in DMSO-d_6 or $CDCl_3$ at constant temperature of 25 °C, and complete assignment was made by means of several 1D and 2D techniques including ^{19}F, *g*COSY, *g*HSQC, and *g*HMQC NMR experiments when needed. ^{1}H and *g*HSQC experiments were collected on a 400 Unity Varian or 500 Inova Varian instrument. ^{13}C and ^{19}F experiments were collected using a 300 Inova Varian instrument. Chemical shifts are reported in parts per million (ppm) downfield from the $CHCl_3$ residual line (δ = 7.27 ppm) or from the DMSO residual line (δ = 2.49 ppm) and were assigned as singlet (s), doublet (d), triplet (t), quartet (q), broad quartet (bq), broad singlet (bs), doublet of doublets (dd), or multiplet (m). Coupling constants (J) are given in Hz. IR spectra were recorded on a Nicolet Magna 760 (Thermo Fisher Scientific) spectrometer. Mass spectra analyses were performed on a VG Platform (Waters, Manchester, U.K.) mass spectrometer operating in positive electrospray ion mode. Analytical thin layer chromatography (TLC) was performed on glass plates (Merck Kieselgel 60 F254). Visualization was accomplished by UV light (254 nm) and I_2. Column chromatography was performed on silica gel (Merck Kieselgel 70−230 mesh). Chemical purity of compounds was assessed by HPLC (> 95%): Zorbax Bonus RP column (dimensions 150 cm × 4.6 mm, 3.5 μm particle size) or validated equivalent. The column temperature was held at 20 °C. Mobile phase A was a 40/60 mixture of H_2O and CH_3OH + 5 mM NH_4HCO_3. Mobile phase B was a 20/80 mixture of H_2O and CH_3OH + 5 mM NH_4HCO_3. A linear gradient from 0% to 100% of mobile phase B over 30 min was used. Flow rate was 1.0 mL/min, and UV detector wavelength was 210 nm. Sample concentration used was typically 0.6 mg/mL with an injection volume of 5 μL.

All reactions were carried out under anhydrous N_2 atmosphere using standard Schlenk techniques. Most chemicals and solvents were analytical grade and used without further purification.

Phenylmethyl 2-(4-fluoro-2-methylphenyl)-4-oxo-3,4-dihydro-1(2*H*)-pyridinecarboxylate (3). To a suspension of magnesium turnings (55.3 kg, 2.3 kmol) in THF (467 L) at room temperature under N_2 atmosphere, a small amount of I_2 (24.6 g, 97 mmol) was added. The mixture was heated to 35−40 °C. To this suspension, a portion (62 L) of a solution of 2-bromo-5-fluorotoluene (357 kg, 1.89 kmol) in THF (953 L) was added. After

the brown color disappeared, the remaining part of the solution was added dropwise over 1 h, maintaining the temperature of the suspension at 60−70 °C. Then the reaction mixture was refluxed for 2 h. The solution of Grignard's reagent was then cooled to 18−20 °C. To a stirred solution of 4-methoxypyridine **1** (123 kg, 1.12 kmol) in dry THF (406 L) was added dropwise a solution of benzyl chloroformate (263 kg, 1.54 kmol) in dry THF (246 L) at −20 °C over 30−50 min (exothermic step). The resulting mixture was stirred at 20−30 °C for 45−60 min. The solution of the Grignard's reagent previously prepared was added dropwise to the suspension over 50−80 min, maintaining the temperature below −15 °C. After the addition was completed the solution was stirred for 1 h at −20 °C. Then 3% HCl (1353 L) was added slowly followed by toluene (2066 L). The organic layer was separated and washed with a 4.5% aqueous solution of $NaHCO_3$ (1845 L). After separation the organic layer was partially concentrated in vacuo. Isopropyl alcohol (1525 L) was added, and the resulting suspension was stirred 1 h at 20−25 °C and then 2 h at 0−5 °C. The solid was isolated by centrifugation, washed with isopropyl alcohol (234 L), and dried in a vacuum oven at 30−35 °C for 10−25 h to give the title compound **3** (329 kg, 86%). ^{1}H NMR (600 MHz, DMSO-d_6): 8.20 (dd, 1H), 7.31 (bs, 3H), 7.19 (bs, 2H), 7.11 (dd, 1H), 7.07 (dd, 1H), 6.94 (dt, 1H), 5.79 (d, 1H), 5.38 (d, 1H), 5.18 (m, 2H), 3.28 (dd, 1H), 2.35 (d, 1H), 2.27 (s, 3H). MS m/z = 340 $[MH]^+$.

2-(4-Fluoro-2-methylphenyl)-4-piperidinone Camphor-10-sulfonate (4). A solution of compound **3** (200 kg, 59 kmol) in isopropyl alcohol (2200 L) was heated to 50 ± 5 °C until the solid was dissolved. A solution of tris(triphenylphosphine)rhodium-(I) chloride (2 kg, 2.2 mol) in isopropyl alcohol (70 L) was added to the reaction mixture. The solution was then hydrogenated at P = 5 bar of H_2 for 5 h at 60 ± 5 °C. The reaction mixture was cooled to 20 ± 5 °C, and a suspension of 5% Pd/C (48 kg) in isopropyl alcohol (200 L) was added. The mixture was hydrogenated at P = 4 ± 1 bar of H_2 for 5 h at 20 ± 5 °C. Then the catalyst was filtered and washed with isopropyl alcohol (500 L). 10-Camphorsulfonic acid (140 kg) was added, and the solution was concentrated to 700 L. Toluene (2200 L) was added and the solution concentrated again to 700 L. Further toluene (1400 L) was added. The solution was seeded, and the obtained slurry was stirred for 10−12 h at 20−21 °C and then for 6−7 h at 2 ± 2 °C. The solid was isolated by centrifugation, washed with toluene (200 L), and dried in a vacuum oven at 30−35 °C for 11−18 h to give title compound **4** (166 kg, 64.3%). ^{1}H NMR (600 MHz, DMSO-d_6): 9.51 (bs, 1H), 9.32 (bs, 1H), 7.70 (dd, 1H), 7.19 (m, 1H), 7.16 (dd, 1H), 4.98 (d, 1H), 3.62 (m, 2H), 2.95 (dd, 1H), 2.90 (m, 1H), 2.89 (d, 1H), 2.63 (m, 1H), 2.55 (m, 1H), 2.53 (m, 1H), 2.40 (d, 1H), 2.37 (s, 3H), 2.24 (m, 1H), 1.94 (t, 1H), 1.85 (m, 1H), 1.80 (d, 1H), 1.28 (m, 2H), 1.03 (s, 3H), 0.74 (s, 3H). MS m/z = 208 $[MH]^+$.

(2*R*)-2-(4-Fluoro-2-methylphenyl)-4-piperidinone L-(+)-Mandelate (5). To a suspension of **4** (310 kg, 0.7 kmol) in CH_2Cl_2 (2170 L), H_2O (700 L) was added. The suspension was stirred until a clear solution was obtained. Then an 8% aqueous solution of $NaHCO_3$ (1364 L) was added and the system stirred for about 30 min. The organic layer was separated, washed with H_2O (700 L), and the solvent was partially evaporated in vacuo followed by addition of isopropyl alcohol (1240 L) and complete evaporation of the solvent. A solution of L-(+)-mandelic acid (99 kg, 0.65 kmol) in isopropyl alcohol (310 L) was added to the organic phase and the resulting suspension stirred for at least 5 h at 20 ± 3 °C. The solid was isolated by centrifugation, washed with isopropanol (208 L), heptane (93 L), and dried in a vacuum oven at 20 ± 3 °C for 14 h to give **5** (117 kg, 46%). ^{1}H NMR (600 MHz, DMSO-d_6): 7.52 (dd, 1H), 7.40 (d, 2H), 7.33 (t, 2H), 7.27 (t, 1H), 7.02 (m, 2H), 4.96 (s, 1H), 4.06 (dd, 1H), 3.32 (ddd, 1H), 2.90 (td, 1H), 2.52 (m, 1H), 2.46 (m, 1H), 2.32 (s, 3H), 2.29 (m, 1H), 2.22 (m, 1H). MS: m/z 208 $[MH]^+$. The enantiomeric purity of compound **5** was determined by chiral HPLC (99.5:0.5 a/a %) by using a column with Chralcel OD-H as stationary phase (25 cm × 4.6 mm i.d., 5 μm, flow = 1 mL/min, T = 30 °C).

3,5-(Bistrifluoromethylbenzyl)methylamine Hydrochloride (7a). To a stirred solution of 3,5-bis(trifluoromethyl)benzaldehyde (15 g, 58.6 mmol) in dry methanol (90 mL) a 8 M solution of $MeNH_2$ in methanol (8.5 mL, 68 mmol) was added. After 50 min KBH_4 (3.3 g, 61.2 mmol) was added. The reaction mixture was stirred for 1 h at room temperature, then concentrated in vacuo to remove methanol, and diluted with ethyl ether (80 mL). Finally, a 1 M solution of HCl in diethyl ether (93 mL) was added. The solid obtained was filtered and washed with diethyl ether (40 mL) to give title compound **6a** (16.34 g, 89.8%) as a white powder. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 9.30 (2H, bs), 8.32 (2H, s), 8.18 (1H, s), 4.31 (2H, s), 2.55 (3H, s).

(1*R*)-1-[3,5-Bis(trifluoromethyl)phenyl]-*N*-methylethanamine L-Malate (7b). To a solution of 3,5-bis-trifluoromethylacetophenone (300 g, 1.17 mol) in MeOH (1120 mL), a solution of $MeNH_2$ 8 M in EtOH (372 mL, 2.98 mol) was added dropwise in 15 min at 25 °C under nitrogen. Then the mixture was stirred for 24 h. $NaBH_4$ was added portionwise over 30 min (27.9 g, 0.74 mol) at 0 °C. A second amount of $NaBH_4$ was added over 30 min (17.1 g, 0.45 mol) and the mixture stirred for a further 1.5 h. The reaction mixture was concentrated in vacuo to small volume. Then it was slowly poured into a mixture of EtOAc (1500 mL) and a 12% aqueous solution of NH_4Cl (1500 mL). The aqueous phase was re-extracted with EtOAc (1500 mL). The organic layers were collected, washed with 13% aqueous solution of NaCl (300 mL), then evaporated in vacuo to obtain (1*R*)-1-[3,5-bis(trifluoromethyl)phenyl]-*N*-methylethanamine (305 g) as a yellow oil, which was dissolved in EtOAc (2960 mL). Then L-malic acid was added portionwise (146.5 g). The suspension was stirred for 2 h at 25 °C and then for 3 h at 0 °C. The suspension was filtered and washed with EtOAc (300 mL). Then the solid was dried under vacuum obtaining crude (1*R*)-1-[3,5-bis-(trifluoromethyl)phenyl]-*N*-methylethanamine (168 g) as a white solid (enantiomeric purity = 96.5/3.5 via capillary zone electrophoresis). To improve chiral purity the solid (264 g) was suspended in ethyl acetate (3430 mL) and then heated to reflux until complete dissolution and then cooled to 3 °C and stirred 90 min. The suspension was filtered, washed with ethyl acetate (264 mL), then dried to obtain title compound (250 g). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 9.21 (3 H, bs), 8.16 (2 H, bs), 8.09 (1 H, bs), 4.26 (1 H, q), 4.02 (1 H, dd), 2.55 (1 H, dd), 2.33 (3 H, s), 2.35 (1 H, dd), 1.45 (3 H, d). 250 g, enantiomeric purity = 100/0 a/a % via GC: Chiralsil-dex CB column (25 m × 0.25 mm i.d., 0.25 μm film thickness, T = 80 °C for 6 min, then 160 °C with a ramp of 10 °C/min, and finally down to 80 °C; flow (helium) = 1.0 mL/min; split ratio 20:1).

(2*R*)-*N*-{[3,5-Bis(trifluoromethyl)phenyl]methyl}-2-(4-fluoro-2-methylphenyl)-*N*-methyl-4-oxo-1-piperidinecarboxamide (8a). To a solution of **5** (25.5 g, 70.9 mmol) in ethyl acetate (382.5 mL) a saturated aqueous solution of $NaHCO_3$ (382 mL) was added, and the resulting mixture was vigorously stirred for 15 min at room temperature. The aqueous layer was separated and re-extracted with ethyl acetate (255 mL), and the collected organics were dried over Na_2SO_4 and concentrated in vacuo to give **5** as free base. To a solution of triphosgene (9.47 g, 31.92 mmol) in ethyl acetate (66 mL) cooled to 0 °C, a solution of **5** as free base in ethyl acetate (135 mL) and triethylamine (24.7 mL, 177 mmol) was added dropwise over 1 h, maintaining the temperature below 10 °C. After the mixture was stirred for 2 h at 20 °C, ethyl acetate (201 mL) and triethylamine (39.5 mL, 283 mmol) were added to the reaction mixture followed by **7a** (33.3 g, 113 mmol) portionwise over 10 min. The mixture was stirred for 2 h at 20 °C and then quenched with a 10% aqueous solution of NaOH (160 mL). The organics were then washed with a 1% aqueous solution of HCl (4 × 160 mL), brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by flash chromatography (cyclohexane/ethyl acetate 9:1) to give pure **8a** (27.3 g, 78%). ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm):

7.97 (1H, s), 7.77 (2H, s), 7.26 (1H, dd), 6.97 (1H, dd), 6.92 (1H, td), 5.24 (1H, t), 4.60 (1H, d), 4.44 (1H, d), 3.58 (2H, m), 2.80 (3H, s), 2.70 (2H, m), 2.50 (2H, m), 2.28 (3H, s).

(2*R*)-*N*-{(1*R*)-1-[3,5-Bis(trifluoromethyl)phenyl]ethyl}-2-(4-fluoro-2-methylphenyl)-*N*-methyl-4-oxo-1-piperidinecarboxamide (8b). To a solution of **7b** (100 g, 280 mmol) in ethyl acetate (400 mL) a 15% aqueous solution of Na_2CO_3 (400 mL) was added. The mixture was stirred at room temperature for 15 min. The organic layer was separated and washed with a 10% aqueous solution of NaCl (400 mL), dried over Na_2SO_4, and concentrated in vacuo to 300 mL to give **7b** as free base. To a solution of **5** (130 g, 320 mmol) in ethyl acetate (500 mL) a 13% aqueous solution of NH_4OH (325 mL) was added, and the resulting mixture was stirred for 15 min. The organic layer was washed with H_2O (4 × 650 mL) and brine (650 mL), dried over Na_2SO_4, and concentrated in vacuo to 300 mL to give **5** as free base. Then to triphosgene (31.4 g, 104 mmol) dissolved in ethyl acetate (250 mL) the previously prepared solution of intermediate **5** was added at 10 °C under N_2 atmosphere, along with triethylamine (89 mL, 638 mmol). After the mixture was stirred for 15 min at room temperature, **7b** as free base and triethylamine (77.6 mL, 640 mmol) were added and the reaction mixture was heated to reflux for 5 h.

Saturated NH_4OH aqueous solution (210 mL) was added. The organic layer was separated and washed with a 5% aqueous solution of H_2SO_4 (4 × 400 mL) and finally brine (400 mL). The organic phase was concentrated in vacuo to 500 mL, isooctane (500 mL) was added, and the solution was concentrated to 500 mL. The resulting slurry was filtered, the solvent was evaporated in vacuo, and the residue was purified by chromatography (cyclohexane/ethyl acetate 8/2) to give title compound **8b** (90.4 g, 65%). ^{1}H NMR (600 MHz, DMSO-d_6): 7.98 (s, 1H), 7.78 (s, 2H), 7.25 (dd, 1H), 6.98 (dd, 1H), 6.89 (dt, 1H), 5.25 (dd, 1H), 5.16 (q, 1H), 3.63 (m, 1H), 3.56 (m, 1H), 2.75 (dd, 1H), 2.68 (dd, 1H), 2.57 (s, 3H), 2.53 (m, 1H), 2.46 (dt, 1H), 2.27 (s, 3H), 1.58 (d, 3H). MS: m/z 505 $[MH]^+$.

General Procedure of the Synthesis of Compounds 9–22: Synthesis of 15 and 16a Presented as Representative Examples. (2*R*,4*S*)-4-(4-Acetyl-1-piperazinyl)-*N*-{[3,5-bis(trifluoromethyl)-phenyl]methyl}-2-(4-fluoro-2-methylphenyl)-*N*-methyl-1-piperidinecarboxamide (15). 1-Acetylpiperazine (6.27 g, 48.89 mmol) was dissolved in acetonitrile (20 mL). Then the intermediate **8a** (12 g, 24.4 mmol) in acetonitrile (270 mL) was added followed by $NaBH(OAc)_3$ (21.5 g, 34.2 mmol). The reaction mixture was stirred at room temperature for 24 h. The reaction was quenched with aqueous saturated $NaHCO_3$ (40 mL) and water (100 mL), and the resulting solution was concentrated in vacuo to about 140 mL. EtOAc (300 mL) was added, and the organic layer was separated. The aqueous layer was extracted with EtOAc (2 × 125 mL). The collected organic layers were washed with brine (200 mL), dried over Na_2SO_4, and concentrated in vacuo to obtain the crude **15** which was purified by flash chromatography (methanol/ethyl acetate 25/75) to give pure **15** (4.1 g). A portion of the pure free base **15** (3.6 g, 6 mmol) was dissolved in ethyl ether (36 mL) cooled to 0 °C. HCl 1 M in ethyl ether is added (6 mL, 6 mol) and the resulting suspension was stirred for 4.5 h at 3 °C, filtered, and dried in vacuo to give **15** (3.24 g, 23.7%). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 11.13 (1H, bs), 7.95 (1H, s), 7.60 (2H, s), 7.26 (1H, m), 6.94 (1H, dd), 6.83 (1H, m), 4.63 (1H, d), 4.38 (2H, m), 4.20 (1H, d), 3.95 (1H, m), 3.60–3.30 (6H, m), 3.08 (2H, m), 2.93 (3H, m), 2.73 (1H, t), 2.36 (3H, s), 2.20 (2H, m), 2.01 (3H, s), 1.93 (1H, m), 1.69 (1H, q).

(2*R*,4*S*)-1′-Acetyl-*N*-{(1*R*)-1-[3,5-bis(trifluoromethyl)phenyl]-ethyl}-2-(4-fluoro-2-methylphenyl)-*N*-methyl-4,4′-bipiperidine-1-carboxamide Methanesulfonate Salt (16a). 1-Acetylpiperazine (3.9 g, 30.5 mmol) was dissolved in acetonitrile (17.7 mL). Then the intermediate **8b** (7.7 g, 15.7 mmol) dissolved in acetonitrile (177 mL) was added followed by $NaBH(OAc)_3$ (13.6 g, 22 mmol).

The reaction mixture was stirred at room temperature under N_2 atmosphere for 24 h and quenched with saturated $NaHCO_3$ (23.1 mL) and water (61.6 mL).

The resulting solution was concentrated in vacuo. EtOAc (208 mL) was added. The organic layer was separated, and the aqueous layer was re-extracted with ethyl acetate (2 × 77 mL). The collected organics layers were washed with brine (2 × 118 mL), dried over Na_2SO_4, and concentrated in vacuo to give crude **16a** which was dissolved in THF (85.4 mL) and mixed with a solution of methanesulfonic acid (0.89 mL, 13.73 mmol) in THF (6.1 mL). The slurry was stirred for 3 h at 0 °C and the solid filtered, washed with THF (15.4 mL), then dried in vacuo for 48 h to give title compound **16a** as a white solid (4.44 g, 41%). ^{1}H NMR (600 MHz, DMSO-d_6): 9.57 (bs, 1H), 7.99 (bs, 1H), 7.68 (bs, 2H), 7.23 (m, 1H), 6.95 (dd, 1H), 6.82 (m, 1H), 5.31 (q, 1H), 4.45 (m, 1H), 4.20 (dd, 1H), 3.99 (m, 1H), 3.56 (m, 1H), 3.47 (m, 3H), 3.37 (m, 1H), 3.15 (m, 1H), 2.96 (m, 1H), 2.87 (m, 1H), 2.80 (t, 1H), 2.74 (s, 3H), 2.36 (s, 3H), 2.30 (s, 3H), 2.13 (m, 1H), 2.08 (m, 1H), 2.10 (s, 3H), 1.87 (m, 1H), 1.73 (m, 1H), 1.46 (d, 3H). MS: m/z 617 $[MH]^+$, as free base.

Receptor Binding Studies. Materials. [^{3}H]SP (1.26 TBq/mmol) was purchased from Amersham Life Science. SP and GR73632 (δ-aminovaleryl,[6] Pro,[9] *N*-Me-Leu[10])-substance P (6–11) were obtained from Bachem. GR205171 was sensitized in the medicinal chemistry laboratories of GSK. All drugs were prepared as 10 mM solution. In binding experiments vs [^{3}H]SP, all competing drugs were serially diluted in the assay buffer. In binding experiments and FLIPR assays, drugs were serially diluted in DMSO to have a final concentration of the solvent equal to 1%. Fluo-4 was purchased from Molecular Probes. Probenecid was purchased from Aldrich.

Binding Experiments. Membranes from Chinese hamster ovary cells stably transfected with human NK_1 receptor (h NK_1-CHO) were prepared essentially as previously described.[29] [^{3}H]SP binding assay was carried out in 96-well plates in a final volume of 400 μL of 50 mM HEPES, pH 7.4, 3 mM $MnCl_2$, and 0.02% BSA. Incubations proceeded at 22 °C for 60 min and displacement experiments were performed by using 0.5 nM [^{3}H]SP. Nonspecific binding was defined by the addition of 1 μM GR205171. Reactions were stopped by filtration through GF/C filtermats using a cell harvester.

Brain cortical samples from one donor were obtained under approved ethical guidelines. [^{3}H]SP and [^{3}H]GR205171 binding experiments in cerebral tissues were carried out in 96-well deep well polypropylene plates. The assay volume of 400 μL consisted of 100 μL of incubation buffer (containing 50 mM HEPES, pH 7.4, 3 mM $MnCl_2$, and 0.02% BSA), 4 μL of DMSO, or increasing concentrations of **16a** dissolved in DMSO (1 pM to 1 μM final concentration). When [^{3}H]SP binding was performed, an amount of 100 μL of [^{3}H]SP (0.5–0.8 nM final concentration) was used in incubation buffer and 200 μL of membrane suspension (0.6 mg of protein) in incubation buffer containing 2 μg/mL leupeptin, 20 μg/mL bacitracin, and 0.5 μM phosphoramidon. When [^{3}H]GR205171 binding was carried out, an amount of 100 μL of [^{3}H]GR205171 (0.8 nM final concentration) was used in incubation buffer and 200 μL of membrane suspension containing 0.14 mg of protein dissolved in incubation buffer with the addition of 2 μg/mL leupeptin, 20 μg/mL bacitracin, and 0.5 μM phosphoramidon. For both radioligands the incubation proceeded at room temperature for 60 min. Nonspecific binding was defined by the addition of cold SP (1 μM) or GR205171 (1 μM) for the gerbil and human cerebral tissues, respectively. The reaction was stopped by rapid filtration through GF/C filtermats presoaked in 0.5% poly-etylenimmine (PEI) using a Brandel M/96R cell harvester. Filters were washed 3 times with 1 mL of ice cold wash buffer (containing 50 mM HEPES, pH 7.4 and 3 mM $MnCl_2$), and radioactivity was counted in a liquid scintillation counter (β counter, Packard). In each experiment, every concentration of displacer was tested in duplicate.

Measurement of $[Ca^{2+}]_i$ Using FLIPR in h-NK_1-CHO Cells. h-NK_1-CHO cells were seeded into black walled clear-bottom 96-well plates (Costar, U.K.) at a density of 60 000 cells per well and cultured overnight. The cells were then incubated for the labeling in the culture medium containing the fluorescent calcium indicator Fluo-4 AM (2 μM), the organic anion transport blocker probenecid (5 mM), and HEPES (20 mM) for 30 min in a humidified atmosphere of 5% CO_2. After being washed with Hanks' balanced salts solution (HBSS) containing 20 mM HEPES and 2.5 mM probenecid (wash buffer), the cells were incubated for 60 min at 37 °C in wash buffer containing 0.02% BSA (assay buffer) either in the absence (control) or in the presence of **16a** and **16b** (1−10 nM). The plates were then placed into a FLIPR (Molecular Devices, Sunnyvale, CA) to monitor cell fluorescence (λ_{ex} = 488 nm, λ_{em} = 510−570 nm) before and after the addition of different concentrations of SP (2 pM to 300 nM) in assay buffer. FLIPR experiments were carried out by using a laser setting of 1.0 W and a 0.4 s CCD (charge coupled device) camera shutter speed.

In Vivo Studies. Pharmacological Studies. All experiments were prereviewed and approved by a local animal care committee in accordance with the guidelines of the "Principles of Laboratory Animal Care" (NIH Publication No. 86-23, revised 1985) and with a project license that was obtained according to Italian law (Art. 7, Legislative Decree No. 116, January 27, 1992), which acknowledges European Directive 86/609/EEC on the care and welfare of laboratory animals.

A characteristic intense foot tapping behavior is invoked by icv administration of selective NK_1 receptor agonists in Mongolian gerbils. This behavior can be selectively antagonized by CNS penetrating NK1 antagonists. The model is robust and is particularly useful, as standard NK_1 receptor ligands recognize the gerbil NK1 receptor with the same affinity as the human NK_1 receptor. Direct icv infusion of a highly selective tachykinin NK_1 receptor agonist, GR73632, in gerbils evokes a vigorous species-specific behavior. This rhythmic hind limb thumping is not induced by agonists for the other tachykinin receptors. This particular action was used to detect the central or peripheral activity of the tested compound. The skull is exposed, and an amount of 5 μL (3 pmol/5 μL of GR73632) is injected by the insertion of a cuffed 25 gauge needle to a depth of 4 mm below bregma, directly into the lateral ventricle. Immediately the animals were individually placed in a clear Perspex observation box. Following 1 min of recovery, the duration of repetitive hind foot tapping is recorded for 3 min, with a maximum possible duration of 180 s. 1, 4, 8, and 24 h before GR73632 administration, at least seven animals each received treatment of vehicle or test compound (10 mL/kg). All treatments were randomly distributed between the five animals of each cage.

In the social interaction test it has been hypothesized that the time spent in active social interaction reflects the level of stress induced in the animals by the environment and the presence of an unfamiliar partner.[20] Clinically effective anxiolytic drugs (e.g., diazepam) increase the interaction exhibited by the two unfamiliar animals, and these anxiolytic-like effects can be distinguished from potential sedative effects (assessed by the locomotor activity as line crossing). On the day of the experiment pairs of male Mongolian gerbils (50−70 g) matched for the treatment condition but unfamiliar to each other were placed in the center of the arena and their social interaction (sniffing, following, grooming the partner, wrestling) was recorded for 5 min. One hour prior to the test animals received vehicle or **16a** (ip 2 mL/kg or po 10 mL/kg; 10 animals for groups) according to a randomized experimental design.

PK Studies. The pharmacokinetics and oral bioavailability of the test compound were determined following iv (as a bolus) and po (by gavage) administration at 0.5 and 1 mg/kg, respectively, in dog (n = 6), cynomolgus monkey (n = 3), and marmoset (n = 3) and at 1 mg/kg in rat (n = 6). Serial blood sampling was applied. The pharmacokinetics was also studied in mouse at doses of 1 mg/kg, iv, and 2 mg/kg, po, using a composite design with three animals per time point (n = 21 for the iv arm; n = 21 for the po arm). In all studies, blood samples were collected at nominal times after dosing, centrifuged to obtain plasma, and then assayed for test compound concentration using a method based on protein precipitation followed by HPLC−MS/MS analysis.

Data Analysis. Data of competition binding experiments on membranes from hNK1-CHO cells were analyzed by using the nonlinear curve-fitting program MicroSAT 5.2.1. For [^{3}H]SP displacement binding experiments, IC_{50} values were obtained constraining the slope factor to unity. IC_{50} values were converted to K_i using the Cheng−Prusoff equation.[30] Data from competition binding experiments on brain homogenates were analyzed using the nonlinear curve-fitting program LIGAND.[31] K_D of radioligands was assessed by elaborating saturation experiments with LIGAND. In FLIPR experiments on h-NK_1-CHO cells, functional responses were measured as fluorescence intensity (FI) produced after receptor stimulation minus basal FI. Curve fitting was determined by nonlinear regression analysis using GraFit, version 4.0.[32] In functional experiments, the potency of compounds **16a**/**b** was determined by using either the Schild's analysis[33] (competitive antagonism) or the Gaddum equation [nonsurmountable antagonism; pA_2 = log(concn ratio^{-1}) − log (antag concn)], where concn ratio represents the ratio between the EC_{50} values of the agonist in the presence and in the absence of the antagonist.

Behavioral data were subjected to one way analysis of variance (ANOVA) followed by Dunnett's test or a paired t test comparing each compound dose with the related vehicle treatment, using GB-stat software (version 6.5, Dynamic Microsystems Inc.). The dose of **16a** inhibiting foot tapping by 50% (ID_{50}) was determined by a linear regression analysis using RS-1 software and is presented with 95% confidence limits.

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