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Non-peptide NK_1 receptor ligands based on the 4-phenylpyridine moiety

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ABSTRACT

The quinoline nucleus of the previously described 4-phenylquinoline-3-carboxamides NK_1 receptor ligands **7** has been transformed into either substituted or azole—(i.e., triazole or tetrazole) fused pyridine moieties of compounds **9** and **10**, respectively, in order to obtain NK_1 receptor ligands showing lower molecular weight or higher hydrophilicity. The program of molecular manipulations produced NK_1 receptor ligands showing affinity in the nanomolar range. In particular, 4-methyl-1-piperazinyl derivative **9j** showed an IC_{50} value of 4.8 nM and was proved to behave as a NK_1 antagonist blocking Sar^9-SP-sulfone induced proliferation and migration of microvascular endothelial cells. Therefore, compound **9j** has been labeled with [^{11}C]CH_3I ($t_{1/2}$ = 20.4 min, β^+ = 99.8%) starting from the corresponding des-methyl precursor **9i** using with a radiochemical yield of about 10% (not decay corrected) and a specific radioactivity >1 Ci/μmol in order to be used as a radiotracer in next PET studies.

1. Introduction

The neurokinin (NK) receptors belong to the family of seven-transmembrane G-protein-coupled receptors (GPCRs) and are classified into three subtypes: NK_1, NK_2 and NK_3. The undecapeptide member of the tachykinin family Substance P (SP) is the endogenous ligand for NK_1.[1] SP interacts with the NK_1 receptor eliciting a wide variety of biological effects. These include smooth muscle contraction, the transmission of pain and stress signals, the induction of neurogenic inflammation, endothelium-dependent vasodilation, and angiogenesis. The first selective non-peptide NK_1 receptor antagonist CP-96,345[2] (**1**, Fig. 1) was discovered by Pfizer medicinal chemists and stimulated a great interest in this area by the pharmaceutical industry. This research effort produced a large number of potent NK_1 receptor antagonists in the past decade.[3] The control of both chemotherapy[4] and post-surgery nausea and vomiting[5] represents the major therapeutic applications of non-peptide NK_1 receptor antagonists. In fact, aprepitant (**2**) was approved by the FDA in 2003 under the brand name Emend® for the prevention of acute and delayed chemotherapy-induced nausea and vomiting (CINV). In 1995, the potent antagonist properties of 4-phenylisoquinolinone and naphthyridinone derivatives **3** were discovered by researchers from Takeda.[6] The studies performed at Takeda produced TAK-637 (**4**),[7] which was reported to be an orally bioavailable antagonist of NK_1 receptor in the intestinal smooth muscles and potentially useful in the treatment of functional bowel diseases such as irritable bowel syndrome (IBS).[8] It is noteworthy that TAK-637 is apparently unrelated to the best known NK_1 receptor antagonists.

The structure of compound **4** was then simplified to obtain axially chiral 2,3,4,5-tetrahydro-6*H*-pyrido[2,3-*b*][1,5]oxazocin-6-one derivative **5**, which confirmed the importance of both *aR* axial chirality and the stacked conformation of the two phenyl moieties for receptor recognition.[9]

In 2006, researchers from Hoffmann-La Roche disclosed the results obtained within a large medicinal chemistry program devoted to the synthesis of novel NK_1 receptor antagonists and culminated in the discovery of netupitant (**6a**) and befetupitant (**6b**).[10]

Early work performed in our laboratory led to the development of a series of 4-phenylquinoline-3-carboxamide derivatives **7** (Fig. 2). Among them, secondary amide **7h** (R_1 = H, R_2 = CH_2OH) showed affinity in the picomolar range for native human NK_1 receptor expressed in astrocytoma UC11MG cells and behaved as an agonist of NK_1 receptor in endothelial cell proliferation, inositol

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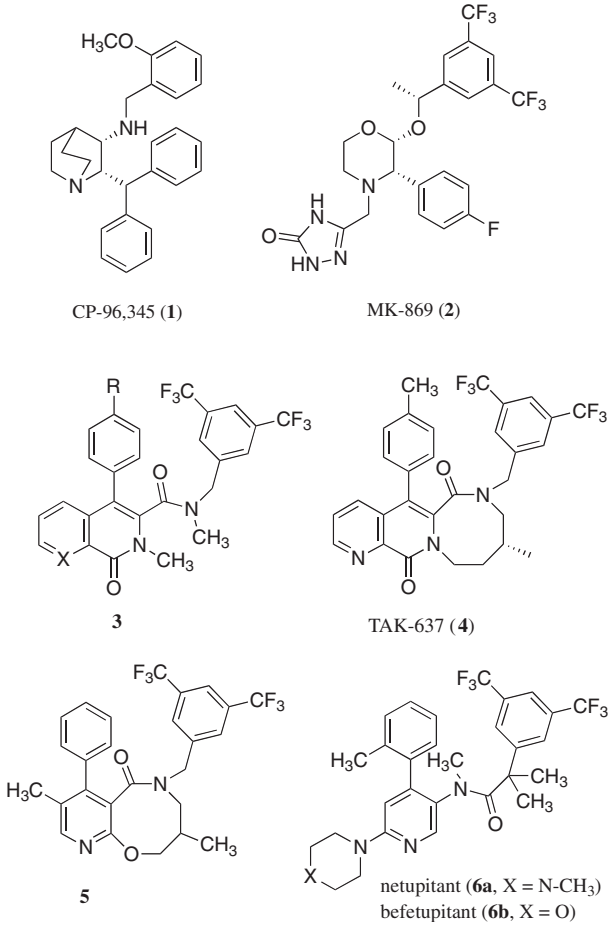


Figure 1. Structures of NK_1 receptor antagonists **1**–**6** from Pfizer, Merck, Takeda, and Hoffmann-La Roche.

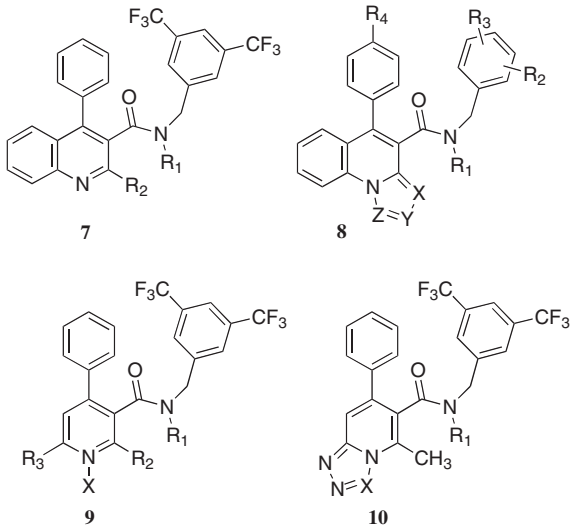
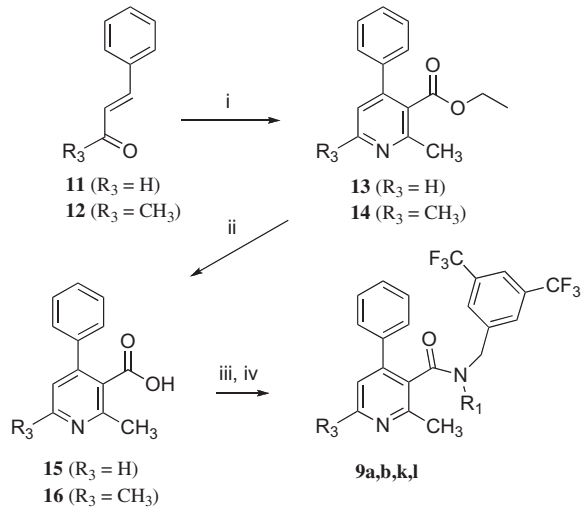
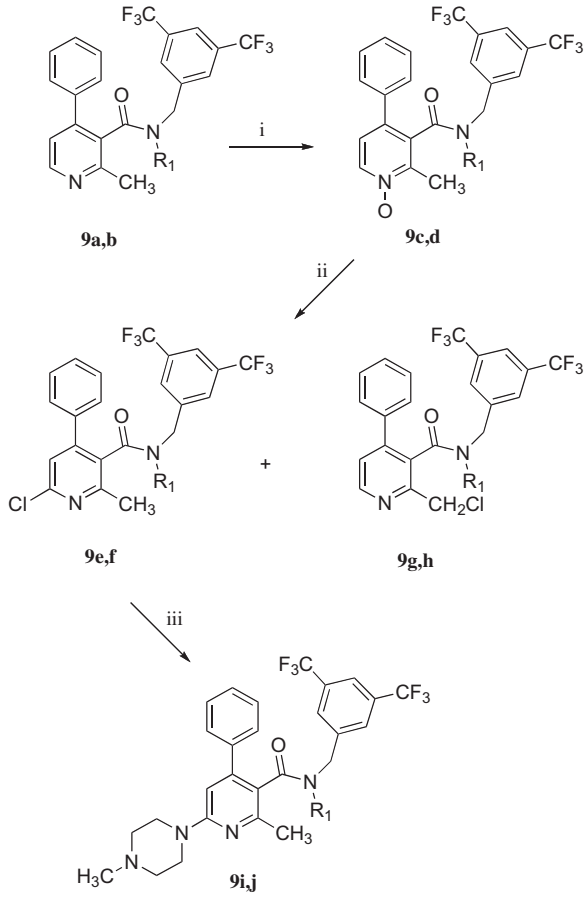


Figure 2. Structures of NK_1 receptor ligands **7**–**10** from our laboratory.



Scheme 1. Reagents: (i) CH_3–$C(NH_2)$=CH–$COOC_2H_5$, piperidine, DMF; (ii) NaOH, H_2O, C_2H_5OH; (iii) $SOCl_2$; (iv) 3,5-$(CF_3)_2C_6H_3CH_2NH_2$, CH_2Cl_2, TEA, or 3,5-$(CF_3)_2C_6H_3CH_2N(H)CH_3$·HCl, CH_2Cl_2, TEA.



Scheme 2. Reagents: (i) *m*-CPBA, $CHCl_3$; (ii) $POCl_3$; (iii) *N*-methylpiperazine.

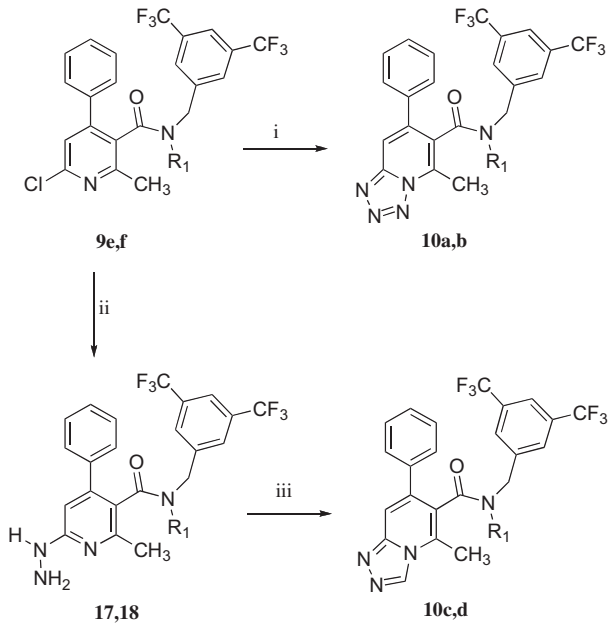
phosphate turnover, and NO-mediated cyclic GMP accumulation, thus proving to be the first non-peptide NK_1 receptor agonist showing very high potency.[11] On the other hand, the corresponding tertiary amide derivative **7i** ($R_1 = CH_3$, $R_2 = CH_2OH$) showed nanomolar affinity for native human NK_1 receptor expressed in astrocytoma UC11MG cells.[11] Subsequently, the results obtained with compounds **7** led to the design of tricyclic carboxamide derivatives **8**, in which the substituent in position 2 of the quinoline nucleus of compounds **7** takes part in a five-membered heterocycle (azole moiety). Within the series of compounds **8**, the tertiary amide derivatives were found to show affinity in the nanomolar range for recombinant human NK_1 receptor, whereas the

corresponding secondary amides were about one order of magnitude less active.[12] Furthermore, the systematic structure variation suggested that electronic features of the tricyclic moiety play a role in modulating the interaction of these amide derivatives with their receptor.[12] As a further step of our large program of structural manipulations, quinoline nucleus of **7** has been transformed into substituted or azole—(i.e., triazole or tetrazole) fused pyridine moieties of compounds **9** and **10**, respectively, in order to obtain NK_1 receptor ligands showing lower molecular weight or higher hydrophilicity.

2. Results and discussion

2.1. Chemistry

The synthesis of 4-phenylpyridine-3-carboxamide derivatives **9** and **10** was carried out by means of the multistep procedure reported in Schemes 1–3. Condensation between ethyl 3-aminocrotonate and the appropriate unsaturated carbonyl derivative (**11** or **12**, Scheme 1) in DMF in the presence of piperidine gave 4-phenylnicotinate derivatives **13**[13] and **14**,[14] which were



Scheme 3. Reagents: (i) NaN_3, DMF; (ii) $NH_2NH_2{\cdot}H_2O$, C_2H_5OH; (iii) HCOOH.

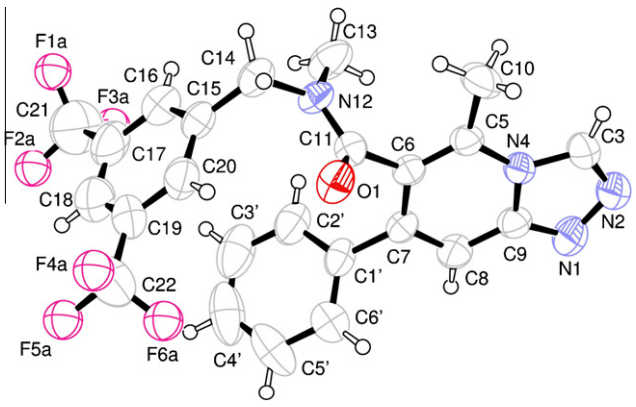
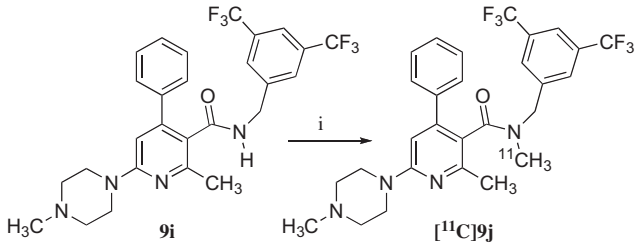


Figure 3. X-ray crystal structure of compound **10d**. Fluorine atoms bound to C21 and to C22 have site occupation factors of 0.61(3) and 0.53(3), respectively.



Scheme 4. Reagents: (i) $[^{11}C]CH_3I$, TBAOH, DMF.

hydrolyzed with 2 M NaOH to give acid derivatives **15** and **16**. Amidation of **15** and **16** under standard conditions provided target amides **9a,b,k,l**.

Compounds **9a** and **9b** were subjected to oxidation with *m*-CPBA (Scheme 2) to obtain the corresponding N-oxides **9c,d**, which were in turn reacted with phosphorus oxychloride to give chloroderivates **9e,f** and **9g,h**. Reaction of imidoyl chlorides **9e,f** with an excess of *N*-methylpiperazine at 130–140 °C gave the target *N*-methylpiperazino derivatives **9i,j** in good yield.

Moreover, the reaction of imidoyl chlorides **9e,f** with sodium azide gave target tetrazolo[1,5-*a*]pyridine derivatives **10a,b** (Scheme 3). On the other hand, the preparation of [1,2,4]triazolo-[4,3-*a*]pyridine derivatives **10c,d** required the two-step procedure shown in Scheme 3, consisting in the reaction of **9e,f** with hydrazine hydrate to obtain hydrazino derivatives **17** and **18**, respectively, which were cyclized with formic acid to obtain the target compounds **10c,d**.

The structure of [1,2,4]triazolo[4,3-*a*]pyridine derivative **10d** was confirmed by X-ray crystallography (Fig. 3). Crystallographic conformation of **10d** shows the two phenyl rings arranged in a parallel, stacked conformation similar to that shown by (*aR*)-**4** in its crystallographic structure,[7] but different from the corresponding one of secondary amide derivative **8a** ($R_1 = R_4 = H$, $R_2 = 3\text{-}CF_3$, $R_3 = 5\text{-}CF_3$, X = Y = Z = CH).[12]

2.2. Radiosynthesis

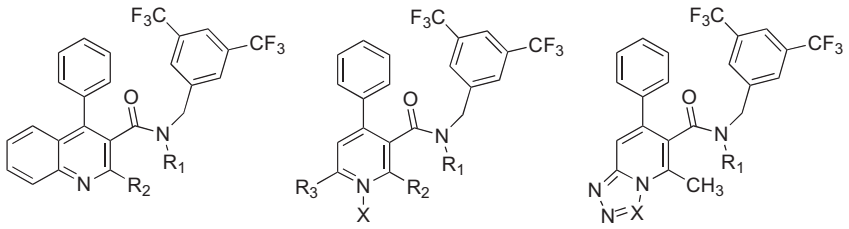
Compound $[^{11}C]$**9j** was synthesized by *N*-[^{11}C]methylation of the corresponding *des*-methyl precursor **9i** with [^{11}C]methyl iodide in the presence of tetrabutylammonium hydroxide (TBAOH) at 80 °C in dimethylformamide (DMF) for 4 min (Scheme 4), with a radioactivity incorporation yield of 65%.

The overall radiosynthesis, including ^{11}C-methylation, HPLC purification and radiopharmaceutical formulation for intravenous administration, was completed in an average time of 35 min from the end of bombardment (EOB), with a radiochemical yield of about 10% (not decay corrected) and a specific radioactivity >1 Ci/μmol at the end of synthesis (EOS). In a typical experiment, starting from ca. 1 Ci of $[^{11}C]CO_2$, 80 mCi of $[^{11}C]$**9j** were obtained with a chemical and radiochemical purity of 95% and 100%, respectively. The identity of the final ^{11}C-radioligand was confirmed by coinjection of the final radiopharmaceutical with its cold standard **9j** on reverse-phase high-performance liquid chromatography (HPLC) (see Section 4).

2.3. Binding studies

Compounds **9a–l** and **10a–d** were tested for their activity in inhibiting the specific binding of [^{125}I]BH-SP to human recombinant NK_1 receptor stably expressed in CHO cells.[15] Owing to the discrepancies observed in the structure–affinity relationships between compounds **8** and **7**,[11,12] some of the previously-published 3-quinolinecarboxamide derivatives **7** were tested in the above test system. The results of the binding studies obtained with 'old

Table 1
Binding affinities of compounds **7**, **9** and **10** for human recombinant NK_1 receptor stably expressed in CHO cells



7 **9** **10**

Compd	R_1	R_2	R_3	X	MW	IC_{50} (nM)[a]
7a	H	H			474.4	208 ± 13
7b	CH_3	H			488.4	92 ± 5.0
7c	H	CH_3			488.4	257 ± 45
7d	CH_3	CH_3			502.4	19 ± 1.2
7h	H	CH_2OH			504.4	227 ± 27
7i	CH_3	CH_2OH			518.5	42 ± 5.2
9a	H	CH_3	H		438.4	653 ± 202
9b	CH_3	CH_3	H		452.6	98 ± 0.20
9c	H	CH_3	H	O	454.4	342 ± 47
9d	CH_3	CH_3	H	O	468.4	32 ± 2.4
9e	H	CH_3	Cl		472.8	270 ± 32
9f	CH_3	CH_3	Cl		486.8	91 ± 8.0
9g	H	CH_2Cl	H		472.8	235 ± 47
9h	CH_3	CH_2Cl	H		486.8	52 ± 8.3
9i	H	CH_3	*N*-Methylpiperazino		536.5	53 ± 11
9j	CH_3	CH_3	*N*-Methylpiperazino		550.5	4.8 ± 1.1
9k	H	CH_3	CH_3		452.4	434 ± 51
9l	CH_3	CH_3	CH_3		466.4	41 ± 6.7
10a	H			N	479.4	279 ± 26
10b	CH_3			N	493.4	57 ± 18
10c	H			CH	478.4	698 ± 130
10d	CH_3			CH	492.4	53 ± 15
SP						1.5 ± 0.10

[a] Each value was calculated from at least three independent experiments performed in duplicate and represents the concentration giving half the maximum inhibition of [^{125}I]SP specific binding to human recombinant NK_1 receptor stably expressed in CHO cells.

compounds' **7a–c,h** (Table 1) confirm the discrepancy between the results obtained with the two test systems. This result suggests the existence of differences between recombinant NK_1 receptors expressed in CHO and native human NK_1 receptors expressed in astrocytoma UC11MG cells. A survey of the literature showed the existence of differences between native and recombinant proteins in a number of different receptors. For instance, human recombinant 5-HT_{1A} receptors expressed in HEK293 cells exist in high and low agonist affinity states, whereas rat cortex 5-HT_{1A} receptors are primarily in the high agonist affinity state.[16] Moreover, researchers from GlaxoSmithKline reported that NK_1 receptor affinity they observed for NKP-608 was intermediate between the corresponding value obtained by Vassourt (pIC_{50} = 7.9) in gerbil and that obtained by Rupniak (pIC_{50} = 10.1) with recombinant human NK_1 receptors.[17] It is noteworthy that secondary amide **7h** showing the highest difference behaves as an agonist. On the contrary, the corresponding tertiary amide **7i** shows similar affinities in the two different test systems. These observations suggest that the integration between binding studies and suitable functional studies is of primary importance in order to correctly characterize the interaction of these amide derivatives with NK_1 receptors.

Because of its determinant role in the interaction with NK_1 receptor binding site,[6,7,12,18] 3,5-bistrifluoromethylbenzyl was kept unmodified in the whole series of compounds **9** and **10**, while the second amide substituent was alternatively a methyl group or a hydrogen atom in order to evaluate the effect of the transformation of the secondary amides in the corresponding tertiary ones. In agreement with the results obtained with compounds **8**, the amide methyl group of tertiary amides **9b**,**d**,**f**,**h**,**j**,**l** and **10b,d** enhances the affinity of the secondary amides **9a**,**c**,**e**,**g**,**i**,**k** and **10a,c** to a variable extent (from 3 to 19 times). The structure–affinity relationships concerning the variation of the heterocyclic moiety, suggest that the human recombinant NK_1 receptor expressed in CHO cells is moderately sensitive to the small structure modulations shown by compounds **9a–h,k,l** and **10a–d**. In particular, the presence of the methyl group in position 2 of the quinoline moiety is beneficial for the NK_1 receptor affinity of tertiary amide **7d** (compare **7d** vs **7b**), while the deletion of its fused benzene ring produces a significant decrease in the receptor affinity (compare **7d** with **9b**). The introduction of a chlorine atom in place of the fused benzene ring as in compound **9f** does not succeed in restoring the original NK_1 receptor affinity (i.e., the one shown by **7d**). Similarly, the N-oxidation (as in compound **9d**), the introduction of a methyl group (**9l**) or the fusion of different azole (e.g., triazole or tetrazole) moieties at *a*-edge of pyridine ring of compound **9b** leads only to small increases in the receptor affinity (compare **9d,l** and **10b,d** vs **9b**), whereas the introduction of a bulkier, basic *N*-methylpiperazine moiety, as in compound **9j**, produces a more significant increase in NK_1 receptor affinity so that the latter compound is the most interesting in the present small series. This result fully agrees with those described by Hoffmann and coworkers[10] and stimulated a deeper biological characterization of **9j**.

It should be noted that the above structure–affinity relationships are roughly (but not perfectly) paralleled in the secondary amide subseries **9a,c,e,g,i,k** and **10a,c**. A more evident dualism in the SAFIR trends between secondary and tertiary amides was observed with compounds **7** using native human NK_1 receptor expressed in astrocytomas UC11MG cells as test system (see Ref. 11).

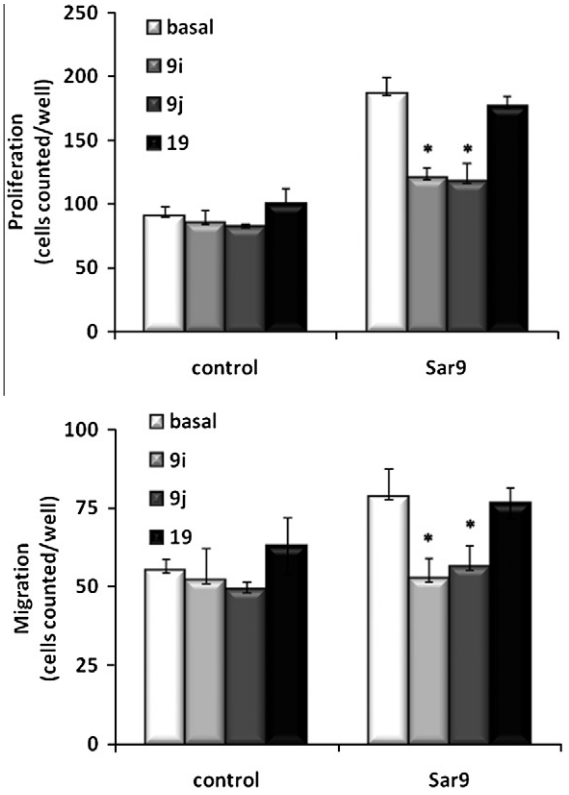


Figure 4. Proliferation (top) and migration (bottom) of CVEC in response to 10 nM Sar^9-SP-sulfone (Sar9): effect of **9i,j**, and **19**. Top panel: Sparse and synchronized endothelial cells were exposed to test substances for 48 h and the number of fixed and stained cells was microscopically counted. Bottom panel: Cell migration toward the NK_1 agonist in the absence/presence of test compounds was evaluated after 4 h of incubation. Data are means ± SEM of three experiments. $^*P$ <0.05 versus agonist alone (Student's *t* test).

Table 2
Inhibition of Sar^9-SP-sulfone induced proliferation and migration. Data are expressed as % inhibition of Sar^9-SP-sulfone induced activity

Compound–dose		Proliferation (% inhibition)	Migration (% inhibition)
9i	1 μM	76.3 ± 6	100 ± 1.4
	100 nM	33.5 ± 4.5	100 ± 8
9j	1 μM	79.7 ± 7	99 ± 3
	100 nM	7.6 ± 3	100 ± 2
19	1 μM	11 ± 6	9 ± 7
	100 nM	0	0

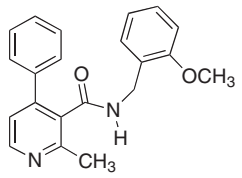


Figure 5. Structure of control substance **19** (see Section 4).

It is remarkable that crystallographic conformation of **10d** shows the two phenyl rings arranged in a parallel, stacked conformation, which appears to be quite similar to that shown by (*aR*)-**4** in its crystallographic structure[7] and is believed to be an important prerequisite for the interaction with NK_1 receptor binding site. The crystallographic result suggests that tertiary amides **9b**,**d**,**f**,**h**,**j**,**l** and **10b,d** are able to adopt the postulated pharmacophorically relevant arrangement.[9] Thus, the comparison between the affinities shown by **10d** and **9j** suggests for *N*-methylpiperazine moiety an important role in the interaction with NK_1 receptor.

2.4. Pharmacological characterization

Since *N*-methylpiperazino derivatives **9i,j** exhibited the highest affinity for the NK_1 receptor, these compounds were selected for a preliminary pharmacological characterization in the same test system used in the study of parent quinoline agonist **7h**.[11] Postcapillary endothelial cells isolated from bovine heart (CVEC) proliferate and migrate when stimulated with NK_1 selective agonists, thus leading to angiogenesis.[11] Compounds **9i** and **9j** were tested on CVEC proliferation and migration and found to be lacking of any agonist activity (Fig. 4).

On the other hand, they completely inhibited the effects induced by NK_1 receptor agonist Sar^9-SP-sulfone (Fig. 4 and Table 2), suggesting that the modification of **7h** leading to **9i,j** abolishes the agonist activity. In the same assays, the negative control substance **19** (Fig. 5, IC_{50} >1000 nM) was devoid of any activity.

While endothelial migration was completely impaired by 100 nM and 1 μM drug concentrations of **9i** and **9j**, the compounds were able to reduce cell proliferation only at 1 μM concentration (Table 2), suggesting that short term biological assay (4 h migration) is more powerful. In conclusion, our results document the activity of compounds **9i** and **9j** as NK_1 antagonists.

3. Conclusions

On the basis of the intriguing results obtained with compounds **7** and **8**, a small series of NK_1 receptor ligands based on the 4-phenylpyridine scaffold was designed, synthesized, and evaluated for its potential ability to inhibit the specific binding of [^{125}I]BH-SP to recombinant human NK_1 receptor in order to obtain compounds possessing lower molecular weight or higher hydrophilicity. The structure–affinity relationship study confirms the higher potency of tertiary amides with respect to the corresponding secondary ones and suggests that the human recombinant NK_1 receptor expressed in CHO cells is moderately sensitive to small structure modulations in the heterocyclic moiety of compounds **9a–h,k,l** and **10a–d**. However, the introduction of a *N*-methylpiperazine moiety in position 2 of pyridine ring produced compound **9j** showing a NK_1 receptor affinity in the low nanomolar range. This result stimulated a deeper biological characterization of **9j**, which was proved to behave as a NK_1 receptor antagonist blocking Sar^9-SP-sulfone induced proliferation and migration of microvascular endothelial cells. Thus, compound **9j** was labeled with ^{11}C ($t_{1/2}$ = 20.4 min, β^+ = 99.8%) starting from the corresponding des-methyl precursor **9i** by using [^{11}C]CH_3I in the presence of tetrabutylammonium hydroxide in DMF with a radiochemical yield of about 10% (not decay corrected) and a specific radioactivity >1 Ci/μmol in order to be used as a radiotracer in next PET studies.

4. Experimental section

4.1. Chemistry

All chemicals used were of reagent grade. Yields refer to purified products and are not optimized. Melting points were determined in open capillaries on a Gallenkamp apparatus and are uncorrected. Merck silica gel 60 (230–400 mesh) was used for column chromatography. Merck TLC plates, silica gel 60 F254 were used for TLC. ^{1}H NMR spectra were recorded at 200 MHz (Bruker

AC200 spectrometer) or at 400 MHz (Bruker DRX-400 AVANCE spectrometer) in the indicated solvents (TMS as internal standard): the values of the chemical shifts are expressed in ppm and the coupling constants (*J*) in Hz. An Agilent 1100 LC/MSD operating with a electrospray source was used in mass spectrometry experiments. Microanalyses were carried out by means of a Perkin–Elmer Series II CHNS/O Analyzer 2400.

4.1.1. Ethyl 2-methyl-4-phenylnicotinate (13)

A mixture of piperidine (1.5 mL, 15.2 mmol) in DMF (130 mL) containing *trans*-cinnamaldehyde **11** (50 mL, 397 mmol) and ethyl 3-aminocrotonate (38 mL, 301 mmol) was heated to reflux for 1 h. The solvent was then evaporated under reduced pressure and the residue was treated with 1 N HCl, neutralized with a 30% solution of NaOH and extracted with EtOAc. The organic layer was dried over Na_2SO_4 and the solvent was removed under reduced pressure. The residue was purified by flash chromatography with petroleum ether–ethyl acetate (2:1) as the eluent to obtain 12 g (yield 17%) of **13**[13] as a yellow oil. ^{1}H NMR (200 MHz, $CDCl_3$): 0.94 (t, *J* = 7.0, 3H), 2.59 (s, 3H), 4.05 (q, *J* = 7.0, 2H), 7.08 (d, *J* = 5.0, 1H), 7.37 (m, 5H), 8.49 (d, *J* = 5.0, 1H). MS (ESI): *m/z* 242 ($M+H^+$).

4.1.2. Ethyl 2,6-dimethyl-4-phenylnicotinate (14)

A mixture of piperidine (0.8 mL, 8.1 mmol), *trans*-4-phenyl-3-buten-2-one **12** (24 g, 164 mmol), ethyl 3-aminocrotonate (13 mL, 103 mmol) in 60 mL of DMF was refluxed for 30 h. The resulting solution was concentrated under reduced pressure and the residue was purified by flash-chromatography with dichloromethane–ethyl acetate (9:1) as the eluent to obtain 4.2 g (yield 16%) of **14**[14] as a pale yellow oil. ^{1}H NMR (200 MHz, $CDCl_3$): 0.95 (t, *J* = 7.3, 3H), 2.54 (s, 3H), 2.58 (s, 3H), 4.05 (q, *J* = 7.3, 2H), 6.98 (s, 1H), 7.35 (m, 5H). MS (ESI): *m/z* 256 ($M+H^+$).

4.1.3. 2-Methyl-4-phenylnicotinic acid (15)

A mixture of **13** (4.0 g, 16.6 mmol) in ethanol (20 mL) with a 2 N NaOH solution (20 mL) was refluxed for 6 h. The volatile was removed under reduced pressure and the residue was diluted with water and acidified with concentrated HCl to pH 5–6. The mixture was stirred at 0–5 °C and the solid obtained by slow precipitation was collected by filtration, washed with *n*-hexane, and dried under reduced pressure to obtain 1.6 g of **15** (yield 45%) as a white solid (mp 233–234 °C). ^{1}H NMR (200 MHz, DMSO-d_6): 2.49 (s, 3H), 7.21 (d, *J* = 5.0, 1H), 7.43 (m, 5H), 8.47 (d, *J* = 5.3, 1H). MS (ESI, negative ions): *m/z* 212 ($M-H^+$).

4.1.4. 2,6-Dimethyl-4-phenylnicotinic acid (16)

A mixture of **14** (1.6 g, 6.3 mmol) in ethanol (20 mL) with a 2 N NaOH solution (20 mL) was refluxed for 18 h. The reaction mixture was concentrated under reduced pressure, diluted with water, and acidified with concentrated HCl. The precipitate was collected by filtration, washed with *n*-hexane, and dried under reduced pressure to obtain 0.54 g of **16** (yield 38%) as a white solid (mp 207–209 °C). ^{1}H NMR (200 MHz, DMSO-d_6): 2.47 (s, 6H), 7.07 (s, 1H), 7.47 (s, 5H). MS (ESI, negative ions): *m/z* 226 ($M-H^+$).

4.1.5. *N*-[3,5-Bis(trifluoromethyl)benzyl]-2-methyl-4-phenylnicotinamide (9a)

A mixture of **15** (0.45 g, 2.1 mmol) in thionyl chloride (3 mL) was heated to reflux for 3 h. The excess of $SOCl_2$ was then removed by azeotropic distillation with toluene and the chloride derivative obtained was quickly dissolved in dichloromethane (15 mL). To the resulting solution, 3,5-bis(trifluoromethyl)benzylamine (1.3 g, 5.3 mmol) and TEA (1.0 mL) were added. The reaction mixture was heated to reflux for 1 h, and the solvent was removed under reduced pressure. Purification of the residue by flash chromatography with ethyl acetate–petroleum ether (2:1) as the eluent afforded 0.68 g (yield 74%) of **9a** as a white solid (mp 161–162 °C). ^{1}H NMR (200 MHz, $CDCl_3$): 2.65 (s, 3H), 4.43 (d, *J* = 5.2, 2H), 5.81 (t, *J* = 5.4, 1H), 7.14 (d, *J* = 4.5, 1H), 7.33 (m, 5H), 7.49 (s, 2H), 7.76 (s, 1H), 8.52 (d, *J* = 4.5, 1H). MS (ESI): *m/z* 439 ($M+H^+$). Anal. ($C_{22}H_{16}F_6N_2O$) C,H,N.

4.1.6. *N*-[3,5-Bis(trifluoromethyl)benzyl]-*N*,2-dimethyl-4-phenylnicotinamide (9b)

A mixture of **15** (0.94 g, 4.4 mmol) in thionyl chloride (10 mL) was heated to reflux for 3 h, and the excess of $SOCl_2$ was removed by azeotropic distillation with toluene. The chloride derivative obtained was quickly dissolved in dichloromethane (25 mL) and to the resulting solution was treated with *N*-[3,5-bis(trifluoromethyl)benzyl]methylamine hydrochloride (1.5 g, 5.1 mmol) and TEA (1.5 mL). The reaction mixture was heated to reflux for 1 h and concentrated under reduced pressure. The residue was partitioned between dichloromethane and water and the organic layer was dried over sodium sulfate and the solvent was removed under reduced pressure. Purification of the residue by flash chromatography with dichloromethane–ethyl acetate (1:1) as the eluent gave 1.4 g (yield 70%) of **9b** as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$; the spectrum of this amide derivative shows the presence of two different rotamers in the ratio of about 8:2; for the sake of simplicity the integral intensities have not been given): 2.44 (s), 2.59 (s), 2.82 (s), 3.80 (d, *J* = 16.0), 4.20 (d, *J* = 16.0), 4.62 (s), 7.13–7.50 (m), 7.59 (s), 7.71 (s), 7.79 (s), 8.56 (d, *J* = 5.1). MS (ESI): *m/z* 453 ($M+H^+$). Anal. ($C_{23}H_{18}F_6N_2O{\cdot}0.25H_2O$) C,H,N.

4.1.7. *N*-[3,5-Bis(trifluoromethyl)benzyl]-2-methyl-4-phenylnicotinamide 1-oxide (9c)

To a solution of **9a** (1.5 g, 3.4 mmol) in 30 mL of chloroform was added *m*-chloroperbenzoic acid (*m*-CPBA) (1.5 g, 8.7 mmol) and the resulting mixture was stirred at room temperature for 4 h. The reaction mixture was washed with a 5% aqueous solution of K_2CO_3 and subsequently with water. The organic layer was dried over sodium sulfate and the solvent was removed under reduced pressure. Purification of the residue by flash chromatography (ethyl acetate) afforded 1.4 g (yield 91%) of **9c** as a white solid (mp 210–212 °C). ^{1}H NMR (200 MHz, $CDCl_3$): 2.21 (s, 3H), 4.48 (d, *J* = 5.8, 2H), 7.04 (d, *J* = 6.7, 1H), 7.27–7.39 (m, 5H), 7.66 (s, 2H), 7.76 (s, 1H), 7.84 (d, *J* = 6.7, 1H), 8.91 (br t, 1H). MS (ESI, negative ions): *m/z* 453 ($M-H^+$). Anal. ($C_{22}H_{16}F_6N_2O_2$) C,H,N.

4.1.8. *N*-[3,5-Bis(trifluoromethyl)benzyl]-*N*,2-dimethyl-4-phenylnicotinamide 1-oxide (9d)

To a solution of **9b** (1.4 g, 3.1 mmol) in 30 mL of chloroform was added *m*-chloroperbenzoic acid (*m*-CPBA) (1.2 g, 6.9 mmol) and the resulting mixture was stirred at room temperature for 4 h. The reaction mixture was washed with a 5% aqueous solution of K_2CO_3 and subsequently with water. The organic layer was dried over sodium sulfate and the solvent was removed under reduced pressure to afford 1.4 g (yield 96%) of **9d** as a pale yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$; the spectrum of this amide derivative shows the presence of two different rotamers in the ratio of about 8:2; for the sake of simplicity the integral intensities have not been given): 2.49 (s), 2.55 (s), 2.58 (s), 2.81 (s), 3.84 (d, *J* = 16.0), 4.25 (d, *J* = 16.0), 4.51 (d, *J* = 14.7), 4.72 (d, *J* = 14.7), 7.14–7.47 (m), 7.58 (s), 7.74 (s), 7.81 (s), 8.36 (d, *J* = 6.9), 8.38 (d, *J* = 6.7). MS (ESI): *m/z* 469 ($M+H^+$). Anal. ($C_{23}H_{18}F_6N_2O_2$) C,H,N.

4.1.9. *N*-[3,5-Bis(trifluoromethyl)benzyl]-6-chloro-2-methyl-4-phenylnicotinamide (9e)

A mixture of **9c** (0.45 g, 0.99 mmol) in 6 mL of $POCl_3$ was refluxed for 3 h. The reaction mixture was then poured into crushed ice, stirred vigorously and extracted with dichloromethane. The organic layer was dried over sodium sulfate and concentrated

under reduced pressure to obtain an oil residue, which was purified by flash chromatography with petroleum ether–ethyl acetate (2:1) as eluent to afford **9e** (0.18 g, yield 38%) as a white solid (mp 143–145 °C). ^{1}H NMR (200 MHz, $CDCl_3$): 2.44 (s, 3H), 4.33 (d, J = 5.9, 2H), 6.44 (t, J = 5.6, 1H), 7.09 (s, 1H), 7.14–7.35 (m, 5H), 7.44 (s, 2H), 7.70 (s, 1H). MS (ESI, negative ions): m/z 471 ($M-H^+$). Anal. ($C_{22}H_{15}ClF_6N_2O$) C,H,N.

4.1.10. *N*-[3,5-Bis(trifluoromethyl)benzyl]-6-chloro-*N*,2-dimethyl-4-phenylnicotinamide (9f)

A mixture of **9d** (1.4 g, 3.0 mmol) in 10 mL of $POCl_3$ was refluxed for 3 h. The reaction mixture was then poured into crushed ice, stirred vigorously and extracted with dichloromethane. The organic layer was dried over sodium sulfate and concentrated under reduced pressure to obtain an oil residue that was purified by flash chromatography with petroleum ether–ethyl acetate (65:35) as eluent to afford **9f** (0.69 g, yield 47%) as a white foam. ^{1}H NMR (200 MHz, $CDCl_3$; the spectrum of this amide derivative shows the presence of two different rotamers in the ratio of about 8:2; for the sake of simplicity the integral intensities have not been given): 2.41 (s), 2.48 (s), 2.76 (s), 3.78 (d, J = 16.1), 4.17 (d, J = 15.9), 4.56 (m), 7.10–7.41 (m), 7.56 (s), 7.66 (s), 7.73 (s). MS (ESI): m/z 487 ($M+H^+$). Anal. ($C_{23}H_{17}ClF_6N_2O$) C,H,N.

4.1.11. *N*-[3,5-Bis(trifluoromethyl)benzyl]-2-(chloromethyl)-4-phenylnicotinamide (9g)

Compound **9g** was obtained in the synthesis of **9e** and was purified by flash chromatography with petroleum ether–ethyl acetate (2:1) as the eluent to obtain a white solid (0.14 g, yield 30%, mp 176–178 °C). ^{1}H NMR (200 MHz, $CDCl_3$): 4.44 (d, J = 6.0, 2H), 4.83 (s, 2H), 5.91 (br t, 1H), 7.26 (d, J = 5.4, 1H), 7.34 (m, 5H), 7.51 (s, 2H), 7.74 (s, 1H), 8.59 (d, J = 5.3, 1H). MS (ESI, negative ions): m/z 471 ($M-H^+$). Anal. ($C_{22}H_{15}ClF_6N_2O$) C,H,N.

4.1.12. *N*-[3,5-Bis(trifluoromethyl)benzyl]-2-(chloromethyl)-*N*-methyl-4-phenylnicotinamide (9h)

Compound **9h** was obtained as a byproduct in the synthesis of **9f**. After purification by flash chromatography with petroleum ether–ethyl acetate (2:1) as the eluent, the title compound was obtained as a yellow oil (0.42 g, yield 29%). ^{1}H NMR (200 MHz, $CDCl_3$; the spectrum of this amide derivative shows the presence of two different rotamers in the ratio of about 85:15; for the sake of simplicity the integral intensities have not been given): 2.36 (s), 2.77 (s), 3.89 (d, J = 16.1), 4.22 (d, J = 16.3), 4.43 (d, J = 14.8), 4.59–4.73 (m), 4.99–5.07 (m), 7.02 (m), 7.25–7.42 (m), 7.62 (s), 7.75 (s), 8.61 (d, J = 5.5). MS (ESI): m/z 487 ($M+H^+$). Anal. ($C_{23}H_{17}ClF_6N_2O$) C,H,N.

4.1.13. *N*-[3,5-Bis(trifluoromethyl)benzyl]-2-methyl-6-(4-methylpiperazin-1-yl)-4-phenylnicotinamide (9i)

A mixture of **9e** (40 mg, 0.085 mmol) in 3 mL of *N*-methylpiperazine was heated at 140 °C for 20 h. The reaction mixture was diluted with ethyl acetate and washed with water. The organic layer was dried over sodium sulfate and the solvent was removed under reduced pressure. Purification of the residue by flash chromatography with ethyl acetate–TEA (8:2) as eluent gave 44 mg (yield 96%) of **9i** as a white foam. ^{1}H NMR (200 MHz, $CDCl_3$): 2.30 (s, 3H), 2.45 (m, 7H), 3.59 (t, J = 4.9, 4H), 4.31 (d, J = 5.9, 2H), 5.70 (br t, 1H), 6.32 (s, 1H), 7.22–7.31 (m, 5H), 7.44 (s, 2H), 7.70 (s, 1H). MS (ESI): m/z 537 ($M+H^+$). Anal. ($C_{27}H_{26}F_6N_4O$) C,H,N.

4.1.14. *N*-[3,5-Bis(trifluoromethyl)benzyl]-*N*,2-dimethyl-6-(4-methylpiperazin-1-yl)-4-phenylnicotinamide (9j)

A mixture of **9f** (0.68 g, 1.4 mmol) in 15 mL of *N*-methylpiperazine was heated at 140 °C for 20 h. The reaction mixture was diluted with ethyl acetate and washed with water. The organic layer was dried over sodium sulfate and the solvent was removed under reduced pressure. Purification of the residue by flash chromatography with ethyl acetate–TEA (8:2) as eluent gave 0.75 g (yield 97%) of **9j** as a yellow oil. ^{1}H NMR (200 MHz, $CDCl_3$; the spectrum of this amide derivative shows the presence of two different rotamers in the ratio of about 75:25; for the sake of simplicity the integral intensities have not been given): 2.33 (s), 2.42 (s), 2.45 (s), 2.50 (t, J = 4.9), 2.73 (s), 3.61 (t, J = 4.9), 3.71 (d, J = 16.1), 4.31 (d, J = 16.1), 4.50 (d, J = 14.9), 4.60 (d, J = 14.8), 6.39 (s), 7.19–7.45 (m), 7.57 (s), 7.69 (s), 7.75 (s). MS (ESI): m/z 551 ($M+H^+$). Anal. ($C_{28}H_{28}F_6N_4O$) C,H,N.

4.1.15. *N*-[3,5-Bis(trifluoromethyl)benzyl]-2,6-dimethyl-4-phenylnicotinamide (9k)

A mixture of **16** (0.20 g, 0.88 mmol) in thionyl chloride (3 mL) was heated to reflux for 4 h. The excess of $SOCl_2$ was then removed by azeotropic distillation with toluene and the chloride derivative obtained was quickly dissolved in dichloromethane (15 mL). To the resulting solution, 3,5-bis(trifluoromethyl)benzylamine (0.50 g, 2.1 mmol) and TEA (1.0 mL) were added. The reaction mixture was heated to reflux for 1 h, and the solvent was removed under reduced pressure. Purification of the residue by flash chromatography with ethyl acetate–petroleum ether (2:1) as the eluent afforded 0.30 g (yield 75%) of **9k** as a white solid (mp 135–136 °C). ^{1}H NMR (200 MHz, $CDCl_3$): 2.54 (s, 3H), 2.60 (s, 3H), 4.39 (d, J = 6.0, 2H), 5.64 (br t, 1H), 6.97 (s, 1H), 7.26–7.36 (m, 5H), 7.46 (s, 2H), 7.73 (s, 1H). MS (ESI): m/z 453 ($M+H^+$). Anal. ($C_{23}H_{18}F_6N_2O$) C,H,N.

4.1.16. *N*-[3,5-Bis(trifluoromethyl)benzyl]-4-phenyl-*N*,2,6-trimethylnicotinamide (9l)

A mixture of **16** (0.26 g, 1.14 mmol) in thionyl chloride (3 mL) was heated to reflux for 4 h, and the excess of $SOCl_2$ was removed by azeotropic distillation with toluene. The chloride derivative obtained was quickly dissolved in dichloromethane (15 mL) and to the resulting solution was treated with *N*-[3,5-bis(trifluoromethyl)benzyl]methylamine hydrochloride (0.40 g, 1.4 mmol) and TEA (1.0 mL). The reaction mixture was heated to reflux for 1 h and concentrated under reduced pressure. The residue was partitioned between dichloromethane and water and the organic layer was dried over sodium sulfate and the solvent was removed under reduced pressure. Purification of the residue by flash chromatography with ethyl acetate–petroleum ether (2:1) as the eluent gave 0.36 g (yield 68%) of **9l** as a colorless oil. ^{1}H NMR (200 MHz, $CDCl_3$; the spectrum of this amide derivative shows the presence of two different rotamers in the ratio of about 8:2; for the sake of simplicity the integral intensities have not been given): 2.42 (s), 2.53 (m), 2.78 (s), 3.77 (d, J = 16.0), 4.19 (d, J = 16.0), 4.58 (s), 6.98–7.47 (m), 7.57 (s), 7.67 (s), 7.74 (s). MS (ESI): m/z 467 ($M+H^+$). Anal. ($C_{24}H_{20}F_6N_2O$) C,H,N.

4.1.17. *N*-[3,5-Bis(trifluoromethyl)benzyl]-5-methyl-7-phenyltetrazolo[1,5-*a*]pyridine-6-carboxamide (10a)

A mixture of **9e** (0.36 g, 0.76 mmol) in anhydrous DMF (15 mL) with sodium azide (0.10 g, 1.5 mmol) was heated at 130 °C for 48 h. The solvent was then removed under reduced pressure and the residue was purified by flash-chromatography with petroleum ether–ethyl acetate (2:1) as eluent to obtain 50 mg (yield 14%) of **10a** as a white solid (mp 219–220 °C). ^{1}H NMR (200 MHz, $CDCl_3$): 2.95 (s, 3H), 4.47 (d, J = 5.9, 2H), 6.59 (t, J = 5.5, 1H), 7.33–7.45 (m, 5H), 7.56 (s, 2H), 7.65 (s, 1H), 7.77 (s, 1H). MS (ESI, negative ions): m/z 478 ($M-H^+$). Anal. ($C_{22}H_{15}F_6N_5O$) C,H,N.

4.1.18. *N*-[3,5-Bis(trifluoromethyl)benzyl]-*N*,5-dimethyl-7-phenyltetrazolo[1,5-*a*]pyridine-6-carboxamide (10b)

A mixture of **9f** (0.30 g, 0.62 mmol) in anhydrous DMF (10 mL) with sodium azide (80 mg, 1.2 mmol) was heated at 130 °C for 48 h. The solvent was then removed under reduced pressure and the residue was purified by flash-chromatography with petroleum ether–ethyl acetate (2:1) as eluent to obtain 60 mg (yield 20%) of **10b** as a white solid (mp 152–154 °C). ^{1}H NMR (200 MHz, $CDCl_3$; the spectrum of this amide derivative shows the presence of two different rotamers in the ratio of about 8:2; for the sake of simplicity the integral intensities have not been given): 2.52 (s), 2.80 (s), 2.96 (s), 2.98 (s), 3.75 (d, J = 16.0), 4.32 (d, J = 16.0), 4.59 (m), 7.21–7.55 (m), 7.59 (s), 7.73 (s), 7.80 (s), 7.85 (s), 7.89 (s). MS (ESI): m/z 494 ($M+H^+$). Anal. ($C_{23}H_{17}F_6N_5O$) C,H,N.

4.1.19. *N*-[3,5-Bis(trifluoromethyl)benzyl]-5-methyl-7-phenyl-[1,2,4]triazolo[4,3-*a*]pyridine-6-carboxamide (10c)

A mixture of **9e** (0.30 g, 0.635 mmol) in ethanol (15 mL) containing an excess of hydrazine hydrate (10 mL) was refluxed for 8 h. The solvent was then removed under reduced pressure, and the residue was diluted with dichloromethane. The organic layer was washed with water and dried over sodium sulfate. After removal of the solvent under reduced pressure, **17** [MS (ESI): m/z 469 ($M+H^+$)] was promptly dissolved in formic acid (10 mL) and the resulting solution was refluxed for 20 h. The excess of formic acid was removed under reduced pressure, and the residue was dissolved in chloroform. The organic layer was washed with water, dried over sodium sulfate, and concentrated under reduced pressure to get a residue that was purified by flash-chromatography with ethyl acetate–TEA (8:2) as eluent to obtain **10c** (30 mg, yield 10%) as a white solid (mp 205–206 °C). ^{1}H NMR (200 MHz, $CDCl_3$): 2.69 (s, 3H), 4.44 (d, J = 5.9, 2H), 6.77 (t, J = 5.8, 1H), 7.26–7.41 (m, 5H), 7.46 (s, 1H), 7.58 (s, 2H), 7.76 (s, 1H), 8.67 (s, 1H). MS (ESI, negative ions): m/z 477 ($M-H^+$). Anal. ($C_{23}H_{16}F_6N_4O$) C,H,N.

4.1.20. *N*-[3,5-Bis(trifluoromethyl)benzyl]-*N*,5-dimethyl-7-phenyl-[1,2,4]triazolo[4,3-*a*]pyridine-6-carboxamide (10d)

A mixture of **9f** (0.20 g, 0.41 mmol) in ethanol (10 mL) containing an excess of hydrazine hydrate (8 mL) was refluxed for 8 h. The solvent was then removed under reduced pressure, and the residue was diluted with dichloromethane. The organic layer was washed with water and dried over sodium sulfate. After removal of the solvent under reduced pressure, **18** [MS (ESI): m/z 483 ($M+H^+$)] was promptly dissolved in formic acid (8 mL) and the resulting solution was refluxed for 18 h. The excess of formic acid was removed under reduced pressure, and the residue was dissolved in chloroform. The organic layer was washed with water, dried over sodium sulfate, and concentrated under reduced pressure to obtain a residue that was purified by flash-chromatography with ethyl acetate–TEA (8:2) as eluent to afford **10d** (61 mg, yield 30%) as a white solid. An analytical sample was obtained by recrystallization from ethyl acetate by slow evaporation (colorless prisms, mp 217–218 °C). ^{1}H NMR (200 MHz, $CDCl_3$; the spectrum of this amide derivative shows the presence of two different rotamers in the ratio of about 9:1; for the sake of simplicity the integral intensities have not been given): 2.48 (s), 2.70 (s), 2.75 (s), 3.64 (d, J = 16.0), 4.32 (d, J = 16.0), 4.44 (d, J = 14.7), 4.63 (d, J = 14.7), 7.22–7.52 (m), 7.58 (s), 7.65 (s), 7.70 (s), 7.78 (s), 8.78 (s), 8.82 (s). MS (ESI): m/z 493 ($M+H^+$). Anal. ($C_{24}H_{18}F_6N_4O$) C,H,N.

4.1.21. *N*-(2-Metoxybenzyl)- 2-methyl-4-phenylnicotinamide (19)

A mixture of **15** (0.30 g, 1.4 mmol) in thionyl chloride (5 mL) was heated to reflux for 3 h. The excess of $SOCl_2$ was then removed by azeotropic distillation with toluene and the chloride derivative obtained was quickly dissolved in dichloromethane (15 mL). To the resulting solution, 2-methoxybenzylamine (0.30 mL, 2.3 mmol) and TEA (1.0 mL) were added. The reaction mixture was heated to reflux for 1 h, and the solvent was removed under reduced pressure. Purification of the residue by flash chromatography with ethyl acetate–petroleum ether (8:2) as the eluent afforded 0.21 g (yield 45%) of **19** as a white solid (mp 124–125 °C). ^{1}H NMR (200 MHz, $CDCl_3$): 2.56 (s, 3H), 3.60 (s, 3H), 4.33 (d, J = 5.9, 2H), 6.11 (t, J = 5.6, 1H), 6.63–7.33 (m, 10H), 8.40 (d, J = 5.2, 1H). MS (ESI): m/z 333 ($M+H^+$). Anal. ($C_{21}H_{20}N_2O_2\cdot H_2O$) C,H,N.

4.2. X-Ray crystallography

A single crystal of **10d** was submitted to X-ray data collection on a Siemens P4 four-circle diffractometer with a graphite monochromated Mo-Kα radiation (l = 0.71069 Å) at 293 K. The structure was solved by direct methods implemented in SHELXS-97 program.[19] The refinements were carried out by full-matrix anisotropic least-squares on F2 for all reflections for non-H atoms by means of SHELXL-97 program.[20]

The fluorine atoms show a statistical disorder and two sites rotated by 60° from one another have been refined for each trifluoromethyl group. The two sets of fluorine atoms bound to C21 have site occupation factors (s.o.f.) of 0.61(3) and 0.39(3) while those bound to C22 have s.o.f. of 0.53(3) and 0.47(3), respectively.

Crystallographic data (excluding structure factors) for the structure in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication no. CCDC 708808. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK; (fax: +44 (0) 1223 336 033; or e-mail: deposit@ccdc.cam.ac.uk).

4.3. Radiochemistry

4.3.1. Radiosynthesis

Reagents and solvents were obtained from Sigma–Aldrich Italia S.p.A. (Milano, Italy) and from ABX (Radeberg, Germany) and were HPLC or ACS grade.

[^{11}C]Carbon dioxide was produced by the ^{14}N(p,α)^{11}C reaction on a IBA Cyclone 18/9 cyclotron, using 18 MeV proton beam at currents 15–30 μA, and trapped in a hollow stainless steel loop, cooled with liquid nitrogen.

[^{11}C]Methyl iodide was substantially synthesized as described by Crouzel[21] involving the reduction of [^{11}C]CO_2 with 0.07 M $LiAlH_4$ to lithium aluminum-[^{11}C]methylate, hydrolysis of this intermediate organometallic complex, iodination of the formed [^{11}C]methanol with hydriodic acid and distillation through an Ascarite–Sicapent purification column. Radiochemical synthesis was performed on the partially modified fully automated synthesis module (PET Tracer Synthesizer, Nuclear Interface Datentechnik GmbH, Münster, Germany) for [^{11}C]-methylation described in detail elsewhere.[22]

For the methylation reaction, [^{11}C]Methyl iodide was transported by a stream of argon (6 mL/min) into the reaction vessel containing 1.0 mg (0.0019 mmol) of *des*-methyl precursor **9i**. The precursor was dissolved in 100 μL of dimethylformamide (ABX) containing 1.0 μL of tetrabutylammonium hydroxide (60% aqueous solution, Fluka) at 80 °C. After 4 min, the reaction mixture was diluted with 1.2 mL of mobile phase (CH_3CN/sodium dihydrogen orthophosphate monohydrate 0.05 M, 60/40 (v/v); pH 7.2) and injected in semipreparative HPLC for the purification of the compound of interest. After this step, the fraction corresponding to [^{11}C]**9j** was collected in 30 mL of sterile water and the product was recovered by solid-phase extraction (SPE) on pre-activated Sep-Pak tC18 plus cartridge (Waters) with 0.7 mL of ethanol.

The purification by semipreparative HPLC was performed with an ACE C18 column (5 μm, 250 × 10 mm, CPS) using a Sykam HPLC

Pump and monitoring with an UV detector K-2001 (Knauer) set at 254 nm, in series with a radiochemical detector, with a flow rate of 5 mL/min. Retention times were 8.5 min for **9i** and 12 min and 14 min for the first and the second peak of the [^{11}C]**9j** rotamers, respectively. The pH of the final solution was neutral.

For the quality control of the final radiopharmaceutical, an X-Terra RP18 analytical column (5 μm, 250 × 4.6 mm, Waters) was used and the chromatographic process was performed with a Waters 1515 isocratic pump and monitored with both UV and Radiochemical Detectors (a Waters 2487 variable-wavelength UV detector in series with a β^+-Bioscan Flow Count detector); for the analysis, a mobile phase of CH_3CN/Sodium dihydrogen orthophosphate 1-hydrate 0.05 M, 60/40 (v:v), pH 7.2 was used, with a wavelength of 254 nm and 1.0 mL/min flow rate. The retention times of the two rotamers of [^{11}C]**9j** were 6.6 min and 7.4 min for the first peak and the second one, respectively. Data collection and HPLC control were performed with the use of the chromatography software package Empower. The identification of the radiolabelled compound was also confirmed by the coinjection of [^{11}C]**9j** with its cold standard (**9j**).

4.3.2. Mass spectrometry characterization of [^{11}C]9j

After the transfer of the collected fraction on the tC18 plus Sep-Pak cartridge, [^{11}C]**9j** (prepared by carrier added radiosynthesis) was recovered by elution with 5.0 mL of ethanol. The peak was collected in the final sterile vial and its identity was confirmed (after decay of radioactivity) by mass spectrometry. An API QStar Pulsar instrument (Applied Biosystems) was used, with an off-line nanospray ionization source and a QqTOF analyzer.

The mass spectrum shows a peak at *m/z* 551.22 corresponding to the protonated molecule $[M+H]^+$, whose structure has been further confirmed by collision-induced decomposition experiments showing the main elimination of 57 u attributed to a methylaziridine.

4.4. In vitro binding assays

4.4.1. Ligands and reagents

Monoiodinated [^{125}I]-Bolton-Hunter reagent labeled SP (2200 Ci/mmol) was purchased from Perkin–Elmer Life Science. Unlabeled SP was purchased from Bachem. Cell culture media, G418, and fetal calf serum were from Invitrogen.

4.4.2. Cell culture

Permanently transfected CHO cells were grown in a mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (1:1) supplemented with 10% fetal calf serum, 100 U/mL penicillin G, 100 mg/mL streptomycin sulfate, and 100 mg/mL G418 (Life Technologies, Inc.) at 37 °C in a humidified atmosphere of 5% CO_2.

4.4.3. Transfection of cells

Wild-type h-NK_1 receptor were transfected in CHO cells using Lipofectin (Life Technologies, Inc.), and stably expressing clones were isolated following selection with 400 mg/mL G418 (Geneticin) after 3–6 weeks.

4.4.4. Radioreceptor binding assays

Enriched plasma membranes from transfected stable cells were prepared as described[15] and stored (2 mg/mL) at −80 °C. The compounds were dissolved into 1 mM DMSO and stored at −20 °C. Radioreceptor binding assays were made in 1 mL reaction mixture containing 50 mM Hepes–Tris pH 7.4, 5 mM $MgCl_2$, 10 mM leupeptin, 0.1 mg/mL bacitracin, 0.1% (w/v) bovine serum albumin, and 2–3 μg of membrane proteins from transfected CHO cells. The concentration of the radiotracer was kept constant at 10 pM in the presence of increasing concentrations of the compounds to be tested. Reactions lasted for 90 min at room temperature and were terminated by rapid filtration into GF/B glass fiber filtering microplates (Filtermate 196; Packard Instruments, Meriden, CT). Filters were washed three times with 1 mL of ice-cold 50 mM Tris–HCl pH 7.4, and allowed to dry for a few hours. The plates were counted in a Top Count (Packard Instruments) after the addition (50 μL) of Microscint 20 (Packard) to each well. The data of the dose–response experiments were analyzed by means of Allfit[23] program to compute the IC_{50} values.

4.5. Pharmacological studies

Bovine post-capillary venular endothelial cells (CVEC) were obtained and cultured as previously described.[24] Cellular proliferation was quantified in subconfluent and synchronized CVEC by total cell number counted per well after 48 h of incubation.[11] Cell migration was evaluated by means of the Neuroprobe microchemotaxis chamber.[25]

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Supplementary data

Supplementary data (analytical data for compounds **9a**–**l**, **10a**–**d**, and **19**) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.02.031. These data include MOL files and InChiKeys of the most important compounds described in this article.

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