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Synthesis and biodistribution of [^{11}C]R116301, a promising PET ligand for central NK_1 receptors

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Abstract—*N*1-(2,6-Dimethylphenyl)-2-(4-{(2*R*,4*S*)-2-benzyl-1-[3,5-di(trifluoromethyl)[*carbonyl*-^{11}C]benzoyl]hexahydro-4-pyridinyl}-piperazino)acetamide ([^{11}C]R116301) was prepared and evaluated as a potential positron emission tomography (PET) ligand for investigation of central neurokinin(1) (NK_1) receptors. 1-Bromo-3,5-di(trifluoromethyl)benzene was converted in three steps into 3,5-di(trifluoromethyl)[*carbonyl*-^{11}C]benzoyl chloride, which was reacted with *N*1-(2,6-dimethylphenyl)-2-{4-[(2*R*,4*S*)-2-benzylhexahydro-4-pyridinyl]piperazino}acetamide providing [^{11}C]R116301 in 45–57% decay-corrected radiochemical yield. The total synthesis time, from end of bombardment (EOB) to the formulated product, was 35 min. Specific activity (SA) was 82–172 GBq/μmol ($n = 10$) at the end of synthesis. *N*1-([4-^{3}H]-2,6-Dimethylphenyl)-2-(4-{(2*R*,4*S*)-2-benzyl-1-[3,5-di(trifluoromethyl)benzoyl]hexahydro-4-pyridinyl}piperazino)acetamide ([^{3}H]R116301) was also synthesized (SA: 467 GBq/mmol). The B_{max} for [^{3}H]R116301 measured in vitro on Chinese hamster ovary cell membranes stably transfected with the human NK_1 receptor was 19.10 ± 1.02 pmol/mg protein with an apparent dissociation constant of 0.08 ± 0.01 nM. Ex vivo, in vivo and in vitro autoradiography studies with [^{3}H]R116301 in gerbils demonstrated a preferential accumulation of the radioactivity in the striatum, olfactory tubercule, olfactory bulb and locus coeruleus. In vivo, the biodistribution of [^{11}C]R116301 in gerbils revealed that the highest initial uptake is in the lung, followed by the liver and kidney. In the brain, maximum accumulation was found in the olfactory tubercules (1.10 ± 0.08 injected dose (ID)/g 20 min post injection (p.i.)) and the nucleus accumbens (1.00 ± 0.12 ID/g 10 min p.i.). Tissue/cerebellum concentration ratios for striatum and nucleus accumbens increased with time due to rapid uptake followed by a slow wash out (1.29 and 1.64, respectively, 30 min p.i.). A tissue to cerebellum ratio of 1.33 and 1.62 was also observed for olfactory bulb and olfactory tubercules, respectively (20 min p.i.). In summary, [^{11}C]R116301 appears to be a promising radioligand suitable for the visualization of NK_1 receptors in vivo using PET.

1. Introduction

Selective, nonpeptide antagonists for tachykinin receptors first became available 10 years ago. Of the three known tachykinin receptors, drug development focused most intensively on the substance P-preferring receptor, neurokinin(1) (NK_1). Although originally studied as potential analgesic compounds, recent clinical and preclinical research has provided evidence that NK_1 receptor antagonists might be important new tools in anxiolytic and antidepressant therapy.[1,2] If confirmed by further controlled clinical studies, this will represent a mechanism of action distinct from all existing antidepressant agents. An NK_1 antagonist, aprepitant, is in clinical use as anti-emetic agent for chemo- and radiotherapy-induced emesis.[3]

*N*1-(2,6-Dimethylphenyl)-2-(4-{(2*R*,4*S*)-2-benzyl-1-[3,5-di(trifluoromethyl)benzoyl]hexahydro-4-pyridinyl}piperazino)acetamide (R116301, Fig. 1), a centrally active NK_1 antagonist, has been evaluated extensively in

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several pharmacological assays in various species, in vitro as well as in vivo.[4–6] It shows high affinity and selectivity for the NK_1 receptor. R116301 exhibits subnanomolar affinity for the human NK_1 receptor (K_i: 0.45 nM) and over 200-fold selectivity towards NK_2 and NK_3 receptors. Since tachykinin receptors are known to exhibit strong species dependence in pharmacology, the NK_1 affinity of R116301 was investigated on tissue membrane preparations from forebrains of guinea pigs, gerbils, ferrets and rats. Relative to its affinity for the human NK_1 receptor, R116301 showed about 10 times lower affinity for the gerbil, ferret and guinea pig NK_1 receptors (K_i: 6.4, 8.3 and 13 nM, respectively) and 200 times lower affinity for the rat NK_1 receptor (K_i: 98 nM). This shows that the rat is not a suitable species for investigation of the biological properties of R116301. In vivo, the compound inhibited substance P-induced peripheral effects (skin reactions and plasma extravasation in guinea pigs) and a central effect (thumping in gerbils) at low doses (0.08-0.16 mg/kg, subcutaneous or intraperitoneal injection), reflecting its high potency as an NK_1 receptor antagonist and excellent brain disposition.

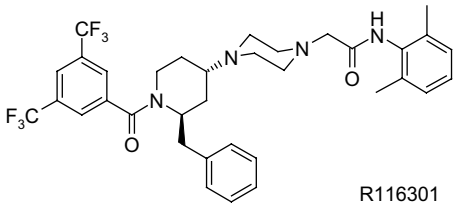


Figure 1. Molecular structure of R116301.

Positron emission tomography (PET) imaging is a technique that can be used to investigate the occupancy of the central receptors by new drugs in function of dose and time, in humans in a noninvasive manner. The receptor occupancy can be evaluated in relation to the therapeutic and side-effects of the drugs. For these reasons *N*1-(2,6-dimethylphenyl)-2-(4-{(2*R*,4*S*)-2-benzyl-1-[3,5-di(trifluoromethyl)[*carbonyl*-^{11}C]benzoyl]hexahydro-4-pyridinyl}piperazino)acetamide ([^{11}C]R116301) was developed.[7] PET experiments with [^{18}F]SPA-RQ in humans have already confirmed the usefulness of labelled antagonists for the quantification of NK_1 receptor occupancy with various doses of NK_1 receptor antagonists.[8] [^{11}C]GR205171 was used successfully for the in vivo characterization of NK_1 receptor binding in rhesus monkey.[9]

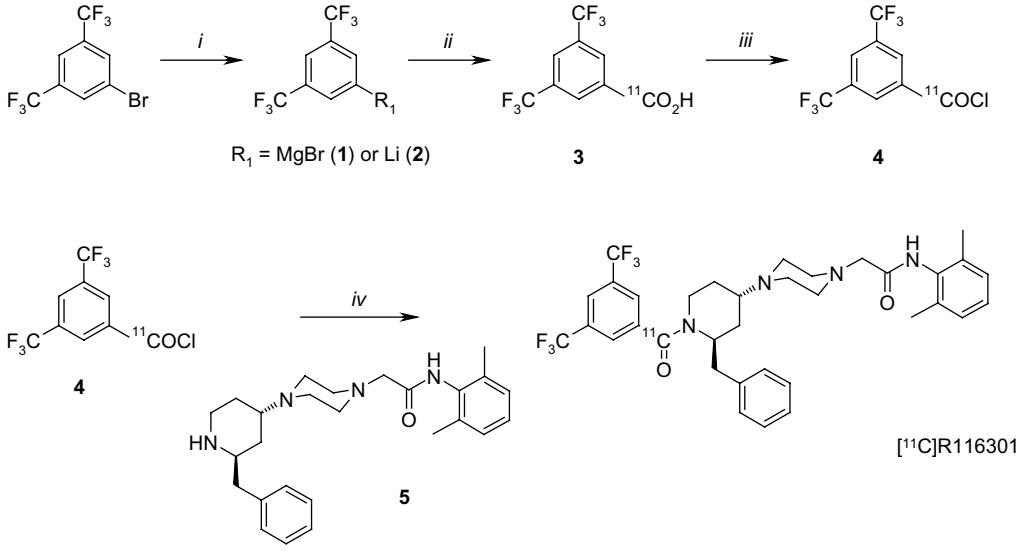
This paper describes the synthesis and pre-clinical evaluation of [^{11}C]R116301. The biodistribution of [^{11}C]R116301 was investigated in gerbils. Furthermore, R116301 was used for saturation radioligand binding analysis and *N*1-([4-^{3}H]-2,6-dimethylphenyl)-2-(4-{(2*R*,4*S*)-2-benzyl-1-[3,5-di(trifluoromethyl)benzoyl]hexahydro-4-pyridinyl}piperazino)acetamide ([^{3}H]R116301) was prepared for examining ex vivo, in vivo and in vitro autoradiography on the gerbil brain.

2. Results and discussion

2.1. Radiosynthesis

A synthesis route was recognized by which a ^{11}C isotope could be introduced converting 1-bromo-3,5-di(trifluoromethyl)benzene in three steps into 3,5-di(trifluoromethyl)[*carbonyl*-^{11}C]benzoyl chloride ([^{11}C]**4**), which was then reacted with *N*1-(2,6-dimethylphenyl)-2-{4-[(2*R*,4*S*)-2-benzylhexahydro-4-pyridinyl]piperazino}acetamide (**5**) providing [^{11}C]R116301 as illustrated in Scheme 1.[10]

2.1.1. 3,5-Di(trifluoromethyl)[*carboxy*-^{11}C]benzoic acid ([^{11}C]3). Synthesis of benzoic acid [^{11}C]**3** via a Grignard reaction was carried out as follows (Scheme 1). First, a 1 M stock-solution of 3,5-di(trifluoromethyl)phenylmagnesium bromide (**1**) in diethyl ether was prepared



Scheme 1. Preparation of [^{11}C]R116301. Reaction conditions: (i) **1**; Mg, diethyl ether **2**; *n*-BuLi, diethyl ether; (ii) 1. [^{11}C]CO_2, 2. HCl, diethyl ether; (iii) oxalyl chloride, DCM; (iv) **5**, DMF, TEA.

from 1-bromo-3,5-di(trifluoromethyl)benzene and magnesium. Next, Grignard reagent **1** was reacted with [^{11}C]CO_2 and subsequently treated with 1 M HCl in diethyl ether affording compound [^{11}C]**3**. The decay-corrected radiochemical yield of compound [^{11}C]**3** was 26 ± 2% (n = 4) at a reaction temperature of 20 °C. A similar reaction was performed with commercially available phenylmagnesium bromide (1 M in tetrahydrofuran (THF)) for comparison. Under the same conditions, [*carboxy*-^{11}C]benzoic acid was obtained in 74 ± 5% yield (n = 4). This large difference in yield is probably caused by diminished reactivity of Grignard reagent **1** in comparison to phenylmagnesium bromide due to the two electron withdrawing trifluoromethyl moieties.

Because of the low yields, compound [^{11}C]**3** was prepared via an alternative synthesis route (Scheme 1). At a temperature of −65 °C, 1-bromo-3,5-di(trifluoromethyl)benzene was converted into the corresponding lithium salt **2** with *n*-butyllithium.[11] Next, [^{11}C]CO_2 was passed through the solution containing derivative **2**, which after the addition of 1 M HCl in diethyl ether, gave compound [^{11}C]**3** in almost quantitative decay-corrected radiochemical yield (97 ± 2%, n = 10).

2.1.2. 3,5-Di(trifluoromethyl)[*carbonyl*-^{11}C]benzoyl chloride ([^{11}C]4). Compound [^{11}C]**3** was transformed into benzoyl chloride [^{11}C]**4** by treatment with oxalyl chloride in dichloromethane (DCM). The conversion yield was >84% (n = 10).

After evaporation of the excess oxalyl chloride at 65 °C, benzoyl chloride [^{11}C]**4** was distilled into another vial containing *N*,*N*-dimethylformamide (DMF). Unfortunately, on average only 60% of the total amount of benzoyl chloride [^{11}C]**4** was collected in the second vial (n = 5). It was therefore decided to focus on developing a one-pot procedure for the synthesis of [^{11}C]R116301.

2.1.3. [^{11}C]R116301. First, the influence of the solvent on the formation of [^{11}C]R116301 was examined. A solution of *N*1-(2,6-dimethylphenyl)-2-{4-[(2*R*,4*S*)-2-benzyl-hexahydro-4-pyridinyl]piperazino}acetamide (**5**) in DCM, THF or DMF including triethylamine (TEA) was added to benzoyl chloride [^{11}C]**4** at 20 °C. DMF was found to be the most suitable solvent resulting in a conversion of 32 ± 4% (n = 4). The conversions obtained with DCM and THF were 0% and 10 ± 2%, respectively (n = 2).

Next, compound **5**, dissolved in DMF and TEA, was added to benzoyl chloride [^{11}C]**4** at various temperatures as summarized in Table 1. Changing the temperature from 20 to 0 °C led to a considerable increase in the [^{11}C]R116301 yield (compare entries 1 and 2, Table 1). Additional heating of the reaction mixture from 0 to 60 and 80 °C proved to be unfavourable due to product decomposition (entry 2 vs entries 3–6, Table 1). Comparison of entries 1, 2, 7 and 8 reveals that the optimal temperature was −20 °C. At this temperature, [^{11}C]R116301 was obtained in a yield of 74 ± 6% starting from benzoyl chloride [^{11}C]**4**.

Table 1. Influence of temperature and time on the synthesis of [^{11}C]R116301

Entry	T (°C)	Time (min)	[^{11}C]R116301[a] (%)
1	20	1	32 ± 4
2	0	1	46 ± 5
3	0 → 60	5	25 ± 4
4	0 → 60	10	20 ± 3
5	0 → 80	5	17 ± 4
6	0 → 80	10	15 ± 3
7	−10	1	59 ± 6
8	−20	1	76 ± 6

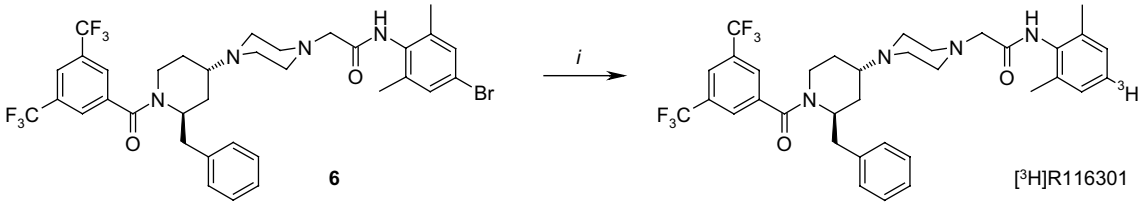
[a] Conversion of benzoyl chloride [^{11}C]**4** into [^{11}C]R116301 ($n \geqslant 4$). Solvent: DMF.

After purification by semipreparative high performance liquid chromatography (HPLC) and isolation by solid phase extraction, [^{11}C]R116301 was produced in an overall decay-corrected radiochemical yield of 45–57% (n = 10). The total synthesis time, from EOB to formulated product, was 35 min. Specific activity was 82–172 GBq/μmol (n = 10) at the end of synthesis.

2.1.4. [^{3}H]R116301. [^{3}H]R116301 was obtained via bromo-tritium exchange from *N*1-(4-bromo-2,6-dimethylphenyl)-2-(4-{(2*R*,4*S*)-2-benzyl-1-[3,5-di(trifluoromethyl)benzoyl]-4-piperidyl}piperazino)acetamide[4] (**6**) as shown in Scheme 2. After purification the resulting [^{3}H]R116301 solution had a radiochemical purity of 98.3%, a specific activity of 467 GBq/mmol and a radiochemical concentration of 15.6 MBq/mL.

2.2. Saturation binding

The binding of [^{3}H]R116301 to membranes of Chinese hamster ovary cells stably transfected with the human NK_1 receptor was saturable and the specific binding displayed a typical hyperbolic saturation curve (Fig. 2). Analysis of the binding data of four independent experiments using iterative nonlinear curve fitting indicated that the [^{3}H]R116301 binding was best fitted to a single



Scheme 2. Preparation of [^{3}H]R116301. Reaction conditions: (i) tritium gas, 10% Pd/C, THF, TEA, 1 h.

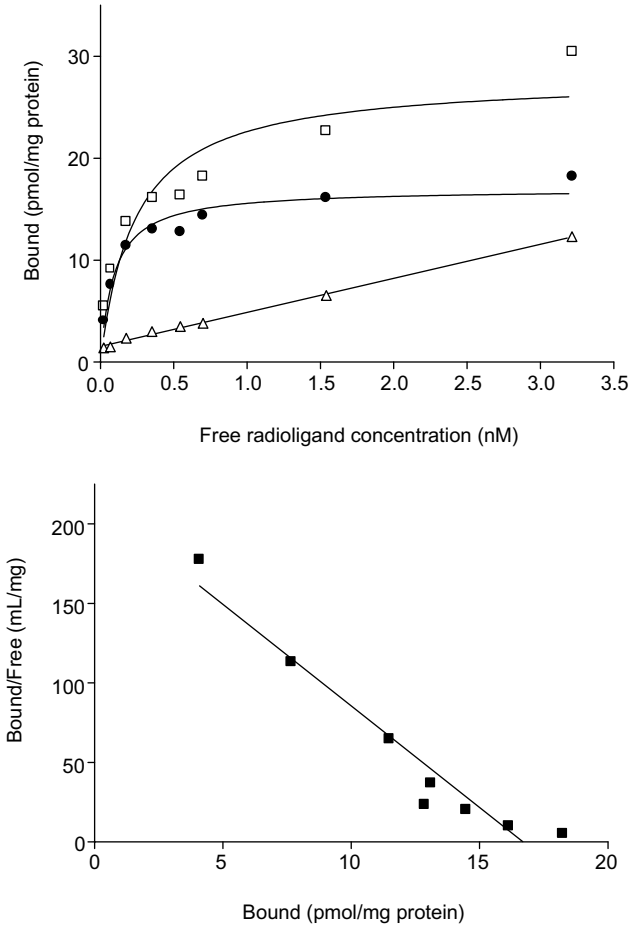


Figure 2. Saturation analysis of the in vitro binding of [^{3}H]R116301 to membranes of Chinese hamster ovary cells stably expressing the human NK_1 receptor. The total (□), specific (●) and nonspecific binding (△) are expressed as a function of the concentration of radiolabelled drug added to the system. Below a Scatchard plot is shown in which the ratio of specifically bound and free drug as a function of the amount of specifically bound drug. Graphs of one representative experiment are shown, the experiment was repeated independently four times, binding parameters were derived by iterative nonlinear curve fitting on the separate saturation curves and averaged; mean values ± SEM ($n = 4$) are: K_d: 0.8 ± 0.01 nM, B_{max}: 19.10 ± 1.02 pmol/mg protein.

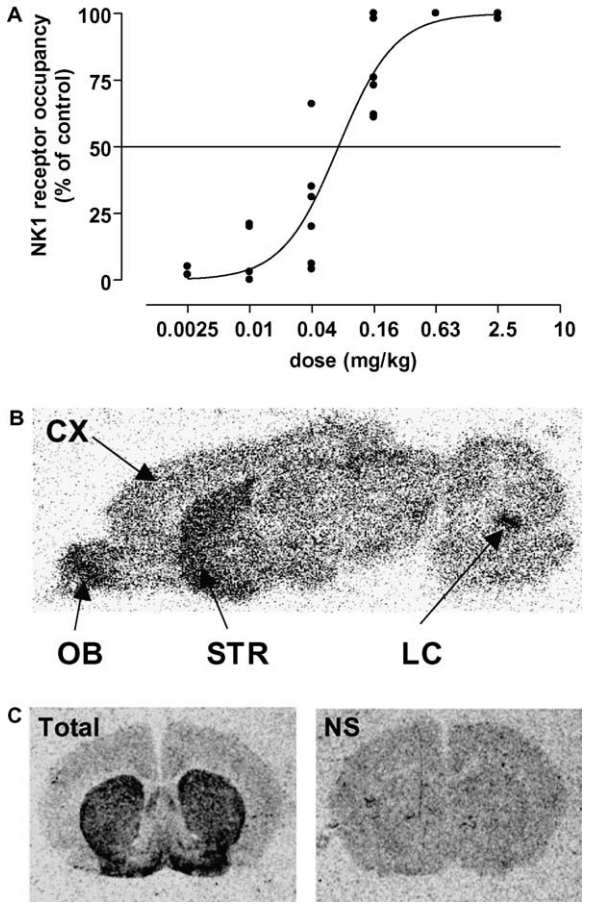


Figure 3. Ex vivo, in vivo and in vitro autoradiographic characterization of R116301. (A) NK_1 receptor occupancy by R116301 in gerbil striatum 1 h after subcutaneous administration measured ex vivo with [^{3}H]-[Sar^9, Met(O$_2$)^{11}]-substance P. (B) In vivo distribution of [^{3}H]R116301 in gerbil brain 1 h after intravenous administration of 370 kBq. STR: Striatum, OB: olfactory bulb, LC: locus coeruleus, CX: cortex. (C) In vitro assessment of the specificity of [^{3}H]R116301 binding in gerbil brain coronal sections at the level of the striatum and olfactory tubercule. Nonspecific binding was measured in presence of 10 μM substance P.

population of sites with $B_{max} = 19.10 \pm 1.02$ pmol/mg protein and a dissociation equilibrium constant of $K_d = 0.08 \pm 0.01$ nM ($n = 4$). These results, together with an extensive pharmacological evaluation reported by Megens et al.,[6] show that R116301 is an NK_1 antagonist showing, saturable and selective binding to the NK_1 receptor. In in vitro and in behavioural studies, mutual antagonism between R116301 and substance P was observed.

2.3. Ex vivo, in vivo and in vitro autoradiography

Individual values and mean curve illustrating the occupancy of NK_1 receptors by R116301 in the gerbil striatum are shown in Figure 3A. Occupancy of NK_1 receptors by R116301 was detectable for dosages $\geqslant 0.04$ mg/kg and was total at dosages $\geqslant 0.63$ mg/kg. The ED_{50} value (95% confidence limit) generated from the curve was 0.07 mg/kg (0.05–0.10). Figure 3B shows that, after intravenous administration, [^{3}H]R116301 is distributed preferentially to NK_1 receptor rich areas such as the striatum, olfactory bulb and locus coeruleus. The specificity of [^{3}H]R116301 binding to NK_1 receptors is illustrated in Figure 3C showing that addition of 10 μM substance P in the incubation medium prevented totally the specific binding of [^{3}H]R116301 to gerbil striatum. The results demonstrate that [^{3}H]R116301 gives good brain penetration and that the binding in NK_1 rich areas is reversible. These are important features of a potential PET ligand upon labelling with ^{11}C. Although Megens et al.[6] describe that R116301 reaches its maximal effect 5 min after intravenous (iv) injection, the gerbils in these experiments were sacrificed 60 min after the iv administration of [^{3}H]R116301 to diminish the amount of nonspecific binding as much as possible. Due to the short half-life of ^{11}C this is less feasible for [^{11}C]R116301.

2.4. Biodistribution experiments in gerbils

Figure 4 summarizes the distribution of [^{11}C]R116301 in the blood, brain and selected peripheral organs 5, 10, 20 and 30 min after the injection. An additional time point of 60 min was also considered. However, since the maximum uptake was already observed at 10 min post injection (p.i.), blood values are very low at 30 min p.i., and the short half-life of ^{11}C it was concluded that the 60 min time point would not contribute any useful data. The uptake of [^{11}C]R116301 is rapid, in most tissues it reaches its maximum concentration at 10 min after tracer injection. The accumulation of radioactivity was highest in the lungs, followed by the liver and kidney. The maximum brain uptake was observed in the olfactory tubercules (1.10 ± 0.08 injected dose (ID)/g 20 min p.i.) and the nucleus accumbens (1.00 ± 0.12 ID/g 10 min p.i.). To determine the target-to-nontarget ratios in the brain, the amount of radioactivity in selected brain regions were compared to that in the cerebellum, an area devoid of NK_1 receptors. As a result of rapid uptake in the striatum and nucleus accumbens and slow clearance from these tissues compared to the cerebellum, the target-to-nontarget ratios increased with time (Fig. 5), which is a strong indication of selective binding to NK_1 receptors. At 30 min after injection, tissue/cerebellum ratios of 1.29 ± 0.02 and 1.64 ± 0.07 were observed for the striatum and nucleus accumbens, respectively. The tissue/cerebellum ratios found for the olfactory bulb and olfactory tubercules reached their corresponding maximum values of 1.35 ± 0.17 and 1.66 ± 0.16 at 20 min p.i. (Fig. 5). In comparison, a similar degree of uptake was displayed in the remainder of the dissected brain areas and cerebellum throughout the time course of the experiment (Fig. 4). A tissue/cerebellum ratio of approximately 1 with no apparent maximum is indicative of nonspecific binding.

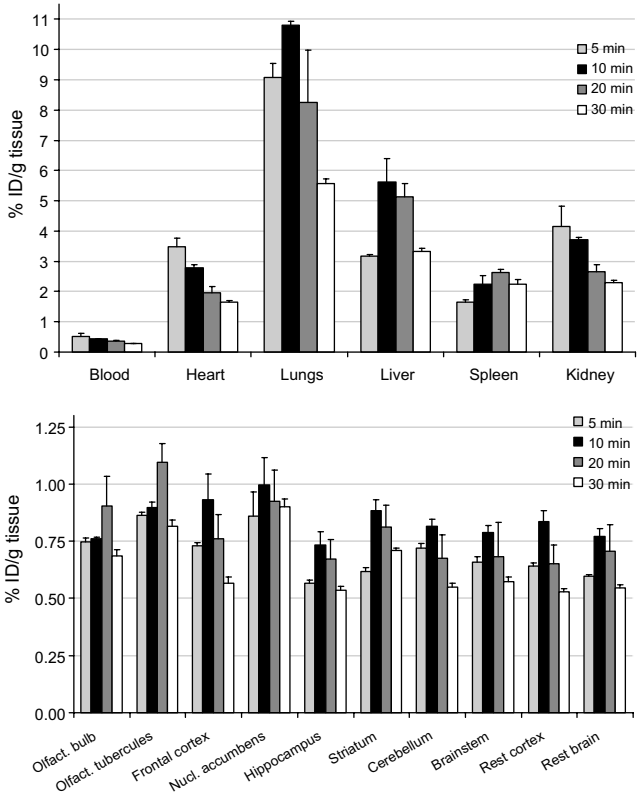


Figure 4. Tissue distribution of radioactivity after intravenous injection of [^{11}C]R116301 in gerbils expressed as % ID/g. Data are means ± SEM (n = 4).

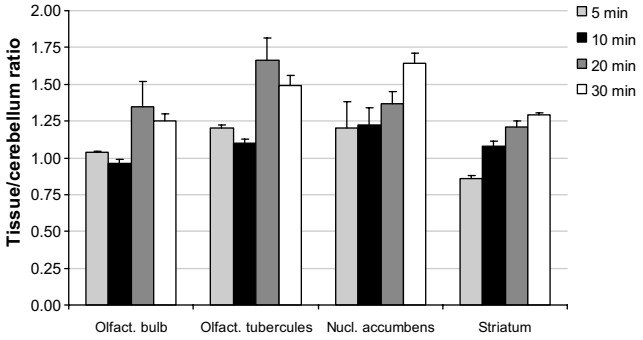


Figure 5. Tissue to cerebellum ratio of [^{11}C]R116301 in gerbil olfactory bulb, olfactory tubercules, nucleus accumbens and striatum. Data are means ± SEM (n = 4).

The results indicate that [^{11}C]R116301 is specifically retained in the areas of high NK_1 receptor density, that is, the striatum, olfactory bulb, tuberculum olfactorium and nucleus accumbens, in agreement with the areas of high NK_1 receptor density detected in radioligand autoradiography on brain sections, where specificity of [^{3}H]R116301 binding is demonstrated by displacement with unlabelled substance P.

3. Conclusion

In summary, [^{11}C]R116301 was synthesized in high radiochemical yield, purity and specific activity by means of a one-pot procedure including 4 (radio)chemical steps. The ex vivo, in vivo and in vitro studies demonstrated that labelled R116301 has high affinity for the NK_1 receptor, sufficiently enters the gerbil brain and accumulates particularly in striatum, olfactory tubercules, nucleus accumbens, olfactory bulb and locus coeruleus, which are demonstrated to be areas of high NK_1 receptor density. [^{11}C]R116301 appears to be a promising radioligand suitable for the visualization of NK_1 receptors in vivo using PET and will be evaluated as such in human volunteers.

4. Materials and methods

4.1. Chemistry

R116301 and the precursors for radiolabelling, compound **5** and **6**, were kindly provided by Johnson and Johnson Pharmaceutical Research and Development (Beerse, Belgium). DMF, TEA, *n*-butyllithium (1.6 M solution in hexane), phenylmagnesium bromide (1.0 M in THF), 1-bromo-3,5-di(trifluoromethyl)benzene, diethyl ether, hydrogen chloride (1.0 and 2.0 M solution in

diethyl ether) and oxalyl chloride (2.0 M solution in DCM) were obtained from Sigma–Aldrich Chemie BV (Zwijndrecht, The Netherlands). Diisopropylamine (DIPA) was purchased from Merck-Schuchardt. Ammonium acetate, acetonitrile (ACN) and methanol (MeOH) were purchased from JT Baker Chemicals B.K. (Deventer, The Netherlands). 10% Pd/C was obtained from Merck and Co. (Darmstadt, Germany). Diethyl ether was freshly distilled from $LiAlH_4$ before use. DMF and TEA were stored over 4 Å molecular sieves. All other chemicals and solvents were used as received unless otherwise stated. Reactions were performed under anhydrous conditions under a He-atmosphere. [^{11}C]Carbon dioxide was produced by the $^{14}N(p,\alpha)^{11}C$ nuclear reaction using an IBA Cyclone 18/9. Semipreparative HPLC was performed with a Jasco PU-1587 preparative 50 mL/min pump (Maarssen, The Netherlands), an in-line Jasco PU-1575 ultraviolet (UV) detector (wavelength 265 nm) and a homemade flow-through radioactivity detector. Jasco-Borwin 1.5 HSS 1500 software was applied for data acquisition and processing. Radioactivity was quantified with a VDC-405 dose calibrator (Veenstra Instruments, Joure, The Netherlands). HPLC for analysis and quality control was performed with a LKB/Pharmacia 2249 pump, an in-line LKB/Pharmacia VWM 2141 UV detector (wavelength 265 nm), a flow-through NaI(Tl) crystal scintillation detection system and Gina-Star version 2.12 (Raytest) for data acquisition and processing. All reactions were carried out in homemade, remotely controlled devices.[12] In the case of tritium labelled R116301, preparative HPLC was performed with only on-line UV detection. For analytical HPLC, radioactivity detection was performed on a Berthold Radioactivity Monitor LB 506 C system with a flow-through cell of 0.500 mL. The eluate was mixed with Pico-Fluor TM 30 (Packard, used as a scintillation cocktail) in an LKB ultrograd mixing unit. Radioactivity of known aliquots was measured by liquid scintillation counting (Packard Tri-Carb 4530), using Ultima Gold (Packard) as a scintillation cocktail.

4.1.1. Radiosynthesis of 3,5-di(trifluoromethyl)-[*carboxy*-^{11}C]benzoic acid ([^{11}C]3)

4.1.1.1. Method A. [^{11}C]CO_2, carried by a stream of helium with a flow of 10 mL/min, was bubbled into a solution of 3,5-di(trifluoromethyl)phenylmagnesium bromide (**1**; 500 μL, 1 M)[13] in diethyl ether. After 4 min, the reaction was quenched by the addition of a hydrogen chloride solution in diethyl ether (500 μL, 1.0 M). An aliquot was checked by analytical HPLC (column: Biorad Aminex ion exclusion HPX-87H, 300 × 7.8 mm; flow: 1.0 mL/min; eluent: ACN/water 1/9 (v/v); λ = 254 nm). Two radioactive peaks corresponding to [^{11}C]CO_2 and compound [^{11}C]**3** were observed (R_t = 6.0 min and R_t = 15.3 min, respectively). The radiochemical yield found for compound [^{11}C]**3** was 26 ± 2% (n = 4, decay-corrected).

A similar experiment was performed with phenylmagnesium bromide (0.5 mL, 1.0 M) for comparison. Both [^{11}C]CO_2 and [*carboxy*-^{11}C]benzoic acid (R_t = 17.3 min) were detected in the reaction mixtures. The radiochemical yield found for [*carboxy*-^{11}C]benzoic acid was 74 ± 5% (n = 4, decay-corrected).

4.1.1.2. Method B. The commercial solvent (hexane) from 1.6 M *n*-butyllithium (10 μL, 160 μmol) was evaporated by bubbling helium through the solution at room temperature (rt). Diethyl ether (300 μL) was added and the reaction vial was cooled to −65 °C. Subsequently, a solution of 1-bromo-3,5-di(trifluoromethyl)benzene (30 μL, 174 μmol) in diethyl ether (50 μL) was added. After 5 min at −65 °C, [^{11}C]CO_2, carried by a stream of helium with a flow of 10 mL/min, was bubbled into the mixture. After 2.5 min, the reaction was quenched by the addition of a hydrogen chloride solution in diethyl ether (160 μL, 160 μmol). The reaction mixture was analyzed by HPLC as described above. Compound [^{11}C]**3** was synthesized in a decay-corrected radiochemical yield of 97 ± 2% (n = 10).

4.1.2. Radiosynthesis of 3,5-di(trifluoromethyl)[*carbonyl*-^{11}C]benzoyl chloride ([^{11}C]4).

After quenching the reaction mixture (see Method B), oxalyl chloride in DCM (160 μL, 320 μmol) was added and the mixture was heated to 50 °C for 3 min in a closed vial. The reaction mixture was cooled to 20 °C in order to reduce the pressure in the reaction vial before opening the exhaust, and then concentrated to dryness under a stream of helium gas by heating at 65 °C. Subsequently, the reaction vial was cooled to 20 °C and ACN was added (1 mL). The resulting solution was analyzed by HPLC (column: Chrompack C18, 10 μm, 250 × 4.6 mm; flow: 1.0 mL/min; eluent: MeOH/H_2O/trifluoroacetic acid 80/20/0.1 (v/v/v); λ = 254 nm). Retention times for compound [^{11}C]**3** and benzoyl chloride [^{11}C]**4** were 5.20 and 6.43 min, respectively. Analogue [^{11}C]**3** was transformed into benzoyl chloride [^{11}C]**4** for >84% (n = 10).

4.1.3. Radiosynthesis of [^{11}C]R116301.

Following the preparation of benzoyl chloride [^{11}C]**4**, a solution of compound **5** (2–3 mg, 4.8–7.1 μmol) in DCM, THF or DMF (200 μL) and TEA (40 μL) was added to the concentrated reaction mixture (see Table 1). Next, the resulting solution was diluted with HPLC solvent (1.5 mL). The reaction mixture was analyzed by HPLC (column: Kromasil C18, 10 μm, 250 × 4.6 mm; flow: 1.0 mL/min; eluent: MeOH/H_2O/DIPA 80/20/0.2 (v/v/v); λ = 265 nm). The maximum conversion of benzoyl chloride [^{11}C]**4** into [^{11}C]R116301 (76 ± 6%; R_t [^{11}C]R116301 = 11.87 min) was observed by adding compound **5** at −20 °C.

Using the optimal reaction conditions for the synthesis of [^{11}C]R116301, the final mixture diluted with HLPC eluent (1.5 mL) was purified by semipreparative HPLC (column: Kromasil C18, 10 μm, 250 × 21 mm; flow: 10 mL/min; eluent: MeOH/H_2O/DIPA 88/12/0.2 (v/v/v), λ = 265 nm). The fraction containing [^{11}C]R116301 (R_t = 11.76 min) was collected in 50 mL of 50 mM sterile NaOH solution and the desired product was isolated by solid phase extraction using a Sep-Pak tC18 plus. After washing with 20 mL of water, the tC18 cartridge was sequentially eluted with 1.3 mL ethanol and 1.5 mL NaCl/NaH_2PO_4 solution. The resulting

solution was filtered through a DLL filter into 12 mL of sterile NaCl/NaH_2PO_4 solution. The decay-corrected radiochemical [^{11}C]R116301 yield was 45–57% ($n = 10$) and chemical and radiochemical purities were >96% as determined by analytical HPLC as described above. Specific activity was between 82 and 172 GBq/μmol ($n = 10$) at the end of synthesis. The overall synthesis time, from EOB to formulated product, was 35 min.

4.1.4. Radiosynthesis of [^{3}H]R116301. A mixture of compound **6** dihydrochloride hydrate (1.25 mg, 1.51 μmol), TEA (5 μL) and 10% Pd/C (5 mg) in dry THF (0.75 mL) was reacted with tritium gas (230 GBq) for 1 h at rt in a closed vessel. Labile tritium was removed, the residue was dissolved in a small amount of EtOH and filtered to remove the catalyst. The solution was concentrated under reduced pressure, the residue was dissolved in DMF (200 μL) and purified by HPLC (column: Kromasil KR 100-10 RP18, 300 × 4.6 mm; flow: 2 mL/min; eluent: ACN/water/DIPA 70/30/0.2 (v/v/v), $\lambda = 220$ nm). The combined product fractions were concentrated in vacuo, coevaporated with EtOH (20 mL) and diluted with EtOH to a final volume of 30.0 mL.

The specific activity (a) and radiochemical purity (b) of the [^{3}H]R116301 solution were determined by HPLC ((a) column: Hypersil ODS, 5 μm, 300 × 4.6 mm; flow: 1.0 mL/min; eluent: water/DIPA 100/0.2 (v/v) to MeOH/DIPA 100/0.2 (v/v) in 30 min, followed by 10 min at the latter conditions; $\lambda = 264$ nm; (b) column: Kromasil KR 100-10 RP18, 300 × 4.6 mm; flow: 2 mL/min; eluent: 1 M ammonium acetate/MeOH/ACN 10/45/45 (v/v/v), $\lambda = 220$ nm). The specific activity was 467 GBq/mmol and the radiochemical purity 98.3%. The radioactivity concentration of the [^{3}H]R116301 solution was 15.6 MBq/mL with a total yield of 468 MBq [^{3}H]R116301 (66% radiochemical yield) as determined by liquid scintillation counting.

4.2. [^{3}H]R116301 binding studies

4.2.1. Cell culture and membrane preparation. Chinese hamster ovary cells were stably transfected with the human neurokinin 1 receptor. The cells were maintained in Dulbecco's modified Eagles medium (DMEM)/Nutrient mixture Ham's F12 (ratio 1:1) supplemented with 10% heat inactivated fetal calf serum and an antibiotic solution containing 100 IU/mL penicillin, 100 μg/mL streptomycin sulfate, 110 μg/mL pyruvic acid and 300 μg/mL L-glutamine. Once a week, the cells are cultured in a selective medium containing 800 μg/mL gentisic acid. The cells were grown in 145 mm petri-dishes until 80–90% confluency. Twenty-four hours before cell collection for membrane preparation, cells were stimulated by adding 5 mM Na-butyrate to the medium. At the day of membrane preparation, the medium was removed and the cells were washed with phosphate buffered saline. The cells were scraped off the plates in 5 mL 50 mM tris(hydroxymethyl)aminomethane hydrochloride (TrisHCl), pH 7.40 using a rubber scraper. They were pelleted by centrifugation at 23,500g for 10 min at 4 °C in a Sorvall-RC 5B centrifuge (DuPont Instruments, Meyvis, Belgium). The pellets were homogenized in 5 mM TrisHCl, pH 7.4 using an Ultra Turrax homogenizer (Janke and Kunkel IKA Labortechnik, Staufen im Breisgau, Germany) and recentrifuged at 29,900g for 20 min at 4 °C. The final pellet was suspended in 50 mM TrisHCl, pH 7.40. Protein concentration was determined following the Bradford procedure. The membranes were divided in 1 mL aliquots and stored at −70 °C.

4.2.2. [^{3}H]R116301 binding. Membranes were thawed on ice and suspended in 50 mM TrisHCl, pH 7.40, supplemented with 1 mM ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA), 2 mM $MgCl_2$ and 0.1% bovine serum albumin (BSA). Membranes were incubated with [^{3}H]R116301 in a final concentration ranging from 0.1 to 4 nM (R116301 was tritiated by Janssen Pharmaceutica, specific activity was 467 GBq/mmol). Nonspecific binding was determined as that remaining in the presence of 1 μM R164461 (*N*,*N*-dimethyl-(5-{[(2*R*,3*S*)-2-({(1*R*)-1-[3,5-di(trifluoromethyl)phenyl]ethyl}oxy)-3-phenyl-1,4-oxazinan-4-yl]methyl}-2*H*-1,2,3-triazol-4-yl)-methanamine hydrochloride). A solution of [^{3}H]R116301, at a concentration of 50 times the final incubation concentration, was made in dimethyl sulfoxide (DMSO). Part of this solution (10 μL) was added to 40 μL buffer. Incubation was started by addition of 400 μL of the membrane suspension and 50 μL of the latter [^{3}H]R116301 solution or blank. The protein concentration in the 0.5 mL final incubation volume was 5.2 μg. Incubation was run for 30 min in 25 °C. Reaction was stopped by addition of 5 mL ice-cold 50 mM TrisHCl pH 7.40 and followed by rapid filtration over GF/B glass fibre filters presoaked in 0.1% polyethylene imine (for at least 1 h) using a 40-well multividor. The filters were washed twice with ice-cold 50 mM TrisHCl pH 7.40 to remove nonbound radioactivity and placed in plastic mini-vials. Ultima Gold MV scintillation fluid (3 mL) was added to each vial. Vials were vigorously shaken and stored for at least 8 h at rt. Thereafter radioactivity was counted in a Packard Tri-Carb 1600 liquid scintillation counter. The counts were expressed in disintegrations per minute (DPM) referring to Packard standard quench curves and internal standards. Saturation binding curves were calculated by computerized nonlinear curve fitting (GraphPadPrism 3 software), using the equation of a rectangular hyperbola. The equilibrium dissociation constant (K_d) and the maximal number of binding sites (B_{max}) were derived (see Fig. 2).

4.3. NK_1 receptor occupancy by R116301 in gerbil brain

Male Mongolian gerbils (40–60 g) were treated by subcutaneous injections of vehicle or R116301 at six dosages ranging from 0.0025 to 2.5 mg/kg body weight (dosages: 0.0025, 0.01, 0.04, 0.16, 0.63, 2.5 mg/kg; 3–6 animals per dose). The animals were decapitated 1 h after drug administration. Brains were immediately removed from the skull and rapidly frozen in dry-ice cooled 2-methylbutane (−40 °C). Twenty micrometres thick coronal sections were cut using a Leica CM 3050 cryostat microtome (van Hopplynus, Belgium) and thaw-mounted on silanized microscope slides (Star

Frost, Knittel Gläser, Germany). The sections were stored at −20 °C until use.

Occupancy of NK_1 receptors was measured in the striatum of each individual gerbil. After thawing, sections were dried under a cold stream of air and then incubated at rt for 10 min with 3 nM [^{3}H]-[Sar^9, Met(O$_2$)^{11}]-substance P (NEN Life Science) in TrisHCl buffer (50 mM, pH 7.4) containing 3 mM $MnCl_2$, 0.3% (w/v) BSA, 40 μg/mL bacitracin, 2 μg/mL chymostatin, 4 μg/mL leupeptin. Nonspecific binding was measured on adjacent sections in the presence of 1 μM substance P. To stop the incubation, the slides were washed (4 × 1 min) in TrisHCl buffer, pH 7.4 at 4 °C followed by a rapid dip in cold distilled water and drying under a stream of cold air.

Quantitative autoradiography analysis was performed after 2 h acquisition with the β-imager (Biospace, Paris) according to a previously published method.[5] The ED_{50} value (dose of drug producing 50% of NK_1 receptor occupancy) was calculated by nonlinear regression analysis, using the GraphPad Prism program (San Diego, Ca).

4.4. In vivo and in vitro autoradiography of [^{3}H]116301 in gerbil brain

In vivo [^{3}H]R116301 (467 GBq/mmol) autoradiography was performed according to the following procedure. [^{3}H]R116301 (370 kBq) was injected intravenously in male Mongolian gerbils (40–60 g). One hour following injection, the gerbils were sacrificed by decapitation, the brains were removed and rapidly frozen in dry-ice cooled 2-methylbutane (−40 °C). Sagital sections, 20 μm thick, were cut using a Leica CM 3050 cryostat microtome (van Hopplynus, Belgium) and thaw-mounted on silanized microscope slides (Star Frost, Knittel Gläser, Germany). After application of copper strips to the back surface of the slides, the slides were placed in the β-imager (Biospace, Paris) for 64 h.

To assess the specificity of [^{3}H]R116301 binding in brain, in vitro autoradiography experiments were performed according to the following procedure. After a 15 min pre-incubation, cryostat-cut sections of gerbil brain were incubated for 90 min at rt in buffer containing 50 mM TrisHCl (pH 7.4), 0.3% (w/v) BSA, 5 mM $MnCl_2$ and 3 nM [^{3}H]R116301. Nonspecific binding was determined by addition of 10 μM substance P to the incubation medium. After incubation, the excess of radioligand was washed off (3 × 10 min) in incubation buffer at 4 °C followed by a rapid dip in cold distilled water. After washing, the slides were dried under a stream of cold air and exposed to Hyperfilm for 6 weeks.

4.5. Biodistribution study in gerbils

[^{11}C]R116301 (82–172 GBq/μmol) was injected intravenously into male Mongolian gerbils (75–100 g). Under diethyl ether anaesthesia the gerbils were sacrificed by cervical dislocation at 5, 10, 20 and 30 min post injection. A blood sample was taken by cardiac puncture and selected tissues were rapidly dissected and weighed. The radioactivity was measured using a LKB Wallac 1282 compugamma CS and the results expressed as percentage of injected dose per gram of tissue (% ID/g). The results are summarized in Figure 4. All animal studies were performed with the approval of the animal experiment ethical committee of the Vrije Universiteit.

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