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[^{11}C]Ro 15-4513, a ligand for visualization of benzodiazepine receptor binding

Preparation, autoradiography and positron emission tomography

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Abstract. Ro 15-4513, a partial inverse agonist at the benzodiazepine (BZ) receptor site was labelled with ^{11}C and used for in vitro autoradiography on human post mortem brain sections and for positron emission tomography (PET) on Cynomolgus monkeys. The total radiochemical yield of [^{11}C]Ro 15-4513 was 30–40% with an overall synthesis time of 40 min. The specific radioactivity was about 1000 Ci/mmol at end of synthesis. In vitro autoradiography showed that [^{11}C]Ro 15-4513 bound specifically predominately in the neocortex of the human brain. Specific binding was also demonstrated in the basal ganglia and the cerebellar cortex. Flumazenil (Ro 15-1788) and clonazepam inhibited the binding in cerebral regions, but a significant proportion in the cerebellum was not inhibited by these agents. This proportion may represent α_6-containing BZ receptors. PET examination of [^{11}C]Ro 15-4513 binding in Cynomolgus monkeys demonstrated high uptake of radioactivity in neocortex. The uptake of radioactivity was markedly displaced by high doses of Ro 15-4513 or clonazepam. [^{11}C]Ro 15-4513 should be a useful ligand to examine BZ receptor characteristics in the living human brain by PET.

Key words: Benzodiazepine receptors – Ro 15-4513 – ^{11}C – Positron emission tomography – PET – In vitro autoradiography – Monkey brain – Human brain

The benzodiazepines (BZ) are well established for their anxiolytic and sedative effects. They interact with the BZ receptor, which is part of the $GABA_A$ receptor and chloride channel complex (Möhler and Okada 1977; Olsen and Venter 1986). BZ receptor agonists potentiate the GABA-induced opening of the chloride channel (Haefely 1990).

The $GABA_A$/BZ receptor complex has been characterized using molecular biology techniques as consisting of multimeric proteins with normally five membrane-spanning subunits (Haefely 1990; Seeburg 1990). Each receptor complex is suggested to consist of two α-subunits, two β-subunits and one γ-, δ-, or ε-subunit (Seeburg 1990). A number of variants of the subunits have been described from cloning studies, and until now six α-subunit variants, three β-subunit variants, two γ-subunit variants and one δ-subunit have been described (Lüddens and Wisden 1991). The pharmacology of BZ receptor binding is assumed to be mainly dependent on the nature of the α-subunit variant.

For studies with positron emission tomography (PET) several radiolabelled benzodiazepine agonists and antagonists have been prepared. Flumazenil (Ro 15-1788) is a selective benzodiazepine receptor antagonist (Fig. 1). [^{11}C]Flumazenil has been used to demonstrate BZ receptor binding in monkeys (Maziere et al. 1983; Ehrin et al. 1984; Hantraye et al. 1984) and man (Persson et al. 1985; Samson et al. 1985; Shinotoh et al. 1986). PET and [^{11}C]flumazenil have also been used to quantify BZ receptor binding in the human brain (Persson et al. 1989), and to demonstrate reduced BZ receptor binding in human epileptic foci (Savic et al. 1988) and in chronic alcohol-dependent patients (Litton et al. 1991).

Ro 15-4513 (Fig. 1) is an azide derivative of flumazenil and has the pharmacological profile of a partial inverse agonist at the $GABA_A$/BZ receptor complex (Polc et al. 1982; Bonetti et al. 1984). Most BZs bind to receptors with any of the subunits α_1, α_2, α_3 and α_5, while Ro 15-4513 has been shown to bind to recep-

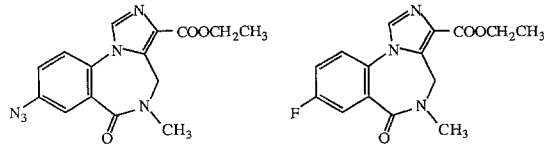

Fig. 1. Structural formula of *left*: Ro 15-4513 (ethyl 8-azido-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a] [1,4]benzodiazepine-3-carboxylate) and *right*: Ro 15-1788 (ethyl 8-fluoro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a] [1,4]benzodiazepine-3-carboxylate)

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tors having the α_6-subunit. This $GABA_A$/BZ receptor complex that have α_6-subunits is mainly present in cerebellar granule cells. [^{3}H]-Labelled Ro 15-4513 has been developed as ligand for the labelling of central BZ receptors in animals in vivo (Sadzot et al. 1989).

Ro 15-4513 has the unique property of antagonizing behavioural and biochemical effects of ethanol (Bonetti et al. 1985, 1989; Fadda et al. 1987; Hoffman et al. 1987). The mechanism underlying this antagonism of ethanol-induced effects is of particular interest to examine in the human brain in vivo. In the present communication [^{11}C]Ro 15-4513 was synthesized and used for in vitro autoradiographic studies on human post mortem brain sections and for in vivo visualization of BZ receptor binding in Cynomolgus monkeys by PET. The specificity and reversibility of [^{11}C]Ro 15-4513 for BZ receptors were examined by displacement studies in vivo using high doses of unlabelled Ro 15-4513 and clonazepam.

Materials and methods

Chemistry

General. Ro 15-4513 and the precursor Ro 44-3902 were kindly supplied by Dr. W. Hunkeler, Hoffman-La Roche, Basle, Switzerland. Other chemicals were obtained from commercial sources and were of analytical grade. [^{11}C]Carbon dioxide was produced at the Karolinska Hospital with a Scanditronix RNP 16 cyclotron using 16 MeV protons in the $^{14}N(p, \alpha)^{11}C$ reaction. The gas target was irradiated in a batch production. The [^{11}C]carbon dioxide produced was trapped in a stainless steel coil cooled with liquid nitrogen before being transferred to the one-pot ^{11}C-alkyl iodide system. [^{11}C]Methyl iodide was synthesized from [^{11}C]carbon dioxide utilizing a one-pot reaction set-up (Halldin et al. 1990).

Semi-preparative reversed-phase HPLC was performed using a Kontron 420 pump, an automatic sample injector (Type VICI with a 1 ml loop), a Waters μ-Bondapak-C18 column (300 × 7.8 mm, 10 μm), and a Kontron 432 UV-detector (wavelength = 254 nm) in series with a GM tube for radiation detection. [^{11}C]Ro 15-4513 was purified using acetonitrile and 0.01 M phosphoric acid (25/75) as the mobile phase with a flow rate of 4.0 ml/min. The radiochemical purity of [^{11}C]Ro 15-4513 was analysed by reversed-phase HPLC using a Kontron 420 pump, a Rheodyne injector (7125 with a 100 μl loop) equipped with a Waters μ-Bondapak-C18 column (300 × 4.6 mm, 10 μm) and an LDC-Milton Roy 300 UV-spectrophotometer (254 nm) in series with a Beckman 170 radioactivity detector. Acetonitrile and 0.01 M phosphoric acid (25/75) was used as the mobile phase with a flow rate of 2.0 ml/min.

[^{11}C]Ro 15-4513 (Fig. 2). [^{11}C]Methyl iodide, prepared as described elsewhere (Halldin et al. 1990), was trapped at room temperature in a reaction vessel (1.0 ml mini-vial, Alltech), containing Ro 44-3902 (1.0 mg), 5 M sodium hydroxide (10 μl) and acetone (300 μl). The vessel was sealed and heated at 80° C for 5 min. Mobile phase (700 μl) was added before injection on to the semi-preparative HPLC column. [^{11}C]Ro 15-4513 eluted after 14–16 min with a retention time identical to a standard reference sample. After evaporation the residue was dissolved in ethanol/propylene glycol (3 ml, 30/70) and sterile phosphate buffer, pH = 7.4 (5 ml) and filtered through a Millipore filter (0.22 μm), yielding a solution which was sterile and free from pyrogens.

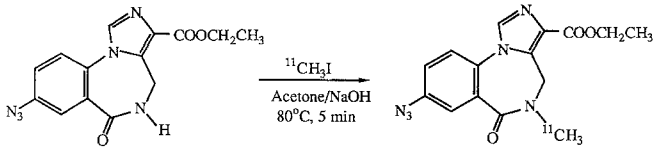

Fig. 2. Synthesis of [^{11}C]Ro 15-4513

In vitro autoradiography

Preparation of brain tissue sections. After approval of the Ethics Committee of the Karolinska Institute human brain tissue was obtained from clinical autopsy at the National Institute of Forensic Medicine, Karolinska Institute, Stockholm, Sweden. After dividing the brain along the midsagittal line, each hemisphere was stored in a plastic bag at −85° C until sectioning (Hall et al. 1988).

To prepare for cryosectioning, the frozen hemisphere was mounted in carboxymethylcellulose (CMC) on a cooled (−70° C) stage. Serial horizontal (canto-meatal) sectioning of the hemisphere block of CMC was performed with a heavy-duty cryomicrotome (LKB 2250, LKB, Stockholm, Sweden). A thin tissue paper was put on the block, after which a transparent tape (3M type 800) was fastened to the block by gentle rubbing. A tissue section (thickness 100 μm) was cut with the microtome and transferred to a cooled, gelatinized glass plate. The tape and the paper were carefully removed, and the section was dried at room temperature. The glass plate with the section was then stored together with dehydrating agents in a refrigerator (+4° C) until use.

Autoradiography procedure. The brain sections were mounted in an incubation chamber (Persson et al. 1991), in which six sections could be incubated separately at each occasion. The sections were incubated for 20 min at room temperature with [^{11}C]Ro 15-4513 and, when applicable, competing unlabelled substance. The specific radioactivity of [^{11}C]Ro 15-4513 was about 600 Ci/mmol corresponding to a concentration of 6.5 nM. After the incubation, the unbound radioactivity was pumped out of the incubation chambers, and the sections were rinsed twice in buffer for 2 min. The sections were then rapidly rinsed in distilled water and then placed on a hot plate (50–70° C) in a warm, dry airflow to facilitate rapid drying.

The dry sections were put into X-ray cassettes together with beta radiation sensitive film (Hyperfilm-βmax, Amersham, UK) for exposure over night. The films were then developed and fixed using conventional photographic techniques.

Analysis of the autoradiograms. The films were analyzed and colour coded using a computerized densitometric system containing a high resolution CCD videocamera and image analysis software (Image 1.31, NIH, Bethesda, MD, USA).

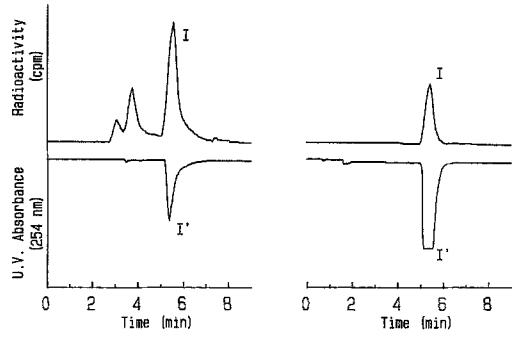

Fig. 3. Analytical HPLC chromatograms (radioactivity and UV vs time). *Left*: before HPLC-purification; *right*: after HPLC-purification. *I*, [^{11}C]Ro 15-4513; *I'*, Ro 15-4513 (added unlabelled reference)

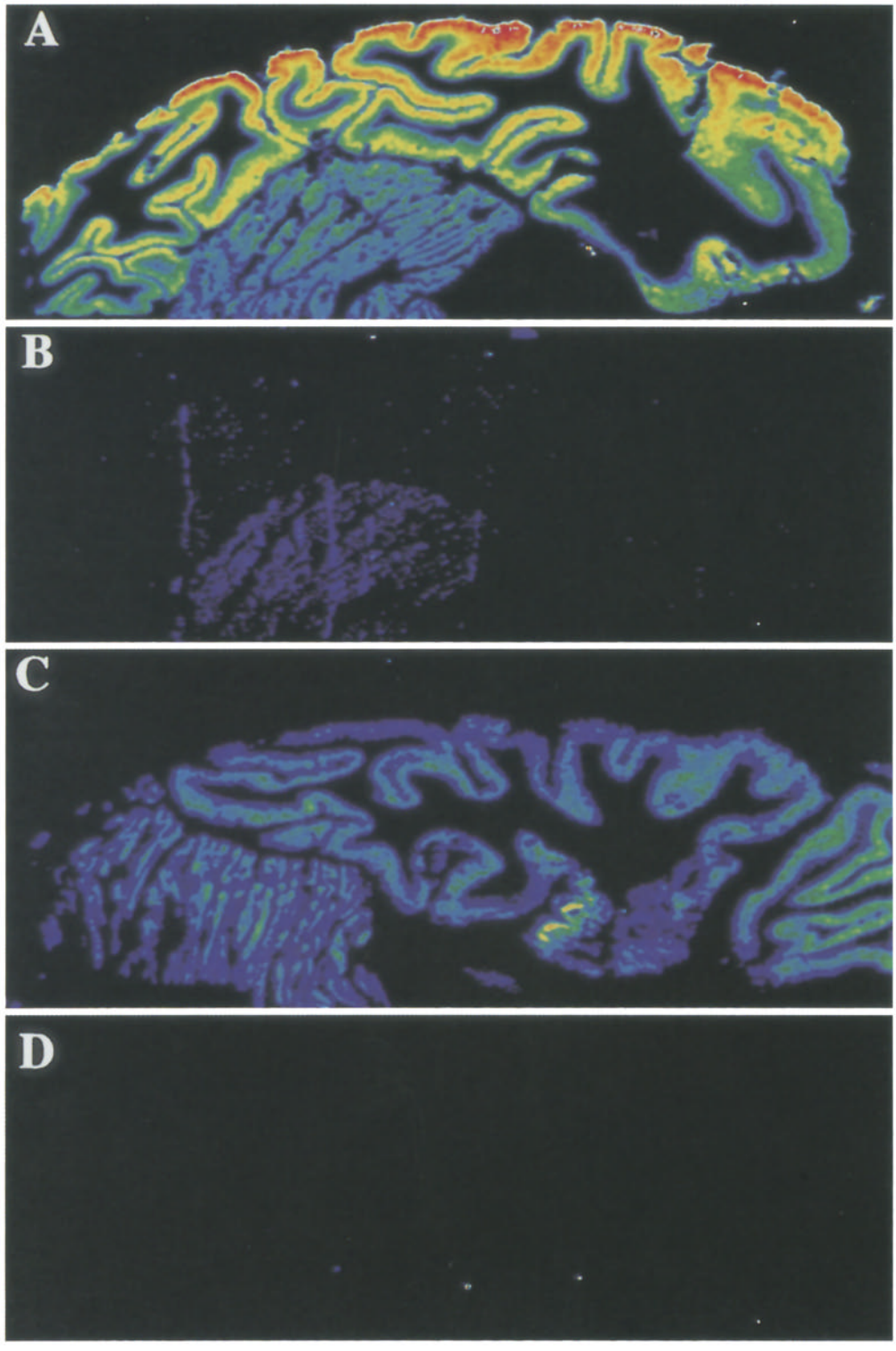

Fig. 4 A–D. Colour coded autoradiograms of canto-meatal human postmortem brain sections showing binding of a tracer concentration (6.5 nM) of [^{11}C]Ro 15-4513 (**A**) without and with the addition of (**B**) flumazenil (1 μM), (**C**) clonazepam (1 μM) or (**D**) Ro 15-4513 (1 μM). The competition experiment with clonazepam was performed at a separate occasion and on a lower level of the brain. The levels of the sections were (**A**) 102.9 mm, (**B**) 103.1 mm, (**C**) 110.4 mm, and (**D**) 103.0 mm from vertex

Positron emission tomography

Positron camera system. The PET-camera system was Scanditronix PC2048-15B (Litton et al. 1990) with a spatial resolution of about 4.5 mm FWHM (full width half maximum). The axial resolution is 6.0 mm FWHM for direct planes and 5.0 mm FWHM for cross planes in the center of the field of view with a slice separation of 6.5 mm. The system measures radioactivity in 15 horizontal slices thus covering the whole extension of the monkey head. Regions of interest were drawn on the reconstructed PET-images. Time-curves were corrected for the radioactive decay of ^{11}C (20.3 min).

PET studies on monkeys. The PET-experiments were performed on two male Cynomolgus monkeys weighing about 5.5 kg. Anaesthesia was induced by repeated im injection of ketamine (Ketalar® 5–10 mg kg^{-1} h^{-1}). A head fixation system was used to secure a fixed position of the monkeys head during the experiments. The

positioning was parallel to the canto-meatal line. [^{11}C]Ro 15-4513 was injected as a bolus into a sural vein. Radioactivity in brain was measured according to a pre-programmed sequence of frames during 1 h after injection of the radioligand. In the first monkey two PET experiments were performed on the same day and 50 MBq [^{11}C]Ro 15-4513 was injected in each experiment. In the first, the regional brain radioactivity of [^{11}C]Ro 15-4513 was followed for 1 h. In the second, performed 3 h later the specificity of the binding was examined by displacement with unlabelled Ro 15-4513. Ro 15-4513 5 mg was injected iv during 2 min starting 19 min after the injection of [^{11}C]Ro 15-4513. In the second monkey the specificity of the binding was further examined. 75 MBq [^{11}C]Ro 15-4513 was injected iv and 19 min later 5 mg of the benzodiazepine receptor agonist clonazepam was administered iv as a bolus during 2 min.

Results

Chemistry

The incorporation of [^{11}C]methyl iodide to [^{11}C]Ro 15-4513 was 60–70% (Fig. 3). The total radiochemical yield of [^{11}C]Ro 15-4513, counted from end of bombardment (EOB) and decay-corrected, was 30–40% with a total synthesis time of 40 min. Purification was performed by semi-preparative reversed-phase HPLC yielding [^{11}C]Ro 15-4513 with a radiochemical purity better than 99% (Fig. 3). To avoid mass-effects between ligand and receptors the specific radioactivity of the radioligand has to be high. The specific radioactivity at end of synthesis (EOS) was about 1000 Ci/mmol (37 GBq/μmol). The specific radioactivities at time for injection of the three administrations of [^{11}C]Ro 15-4513 were 610, 950 and 770 Ci/mmol (22.6, 35.2 and 28.5 GBq/μmol), corresponding to a dose injected of 0.72, 0.46 and 0.85 μg, respectively.

In vitro autoradiography

On the human post mortem brain sections [^{11}C]Ro 15-4513 bound conspiciously to most regions containing grey matter (Fig. 4A). The highest density of [^{11}C]Ro 15-4513 binding was demonstrated in the neocortical regions, but labelling was also evident in the basal ganglia and in the cerebellum. The binding was very low in pons, and virtually no radioactivity was found in white matter.

Addition of a high concentration (1 μM) of unlabelled Ro 15-4513 inhibited the binding of [^{11}C]Ro 15-4513 completely in all regions (Fig. 4D). The addition of flumazenil (1 μM) (Fig. 4B) inhibited the binding of [^{11}C]Ro 15-4513 in most regions but left some binding in the cerebellum. Assuming an approximate linear relationship between film blackness and concentration of radioligand in the tissue, the binding of [^{11}C]Ro 15-4513 which remained in cerebellum after blockade with flumazenil (1 μM) was 30–50%. This is in line with the quantitative data obtained using [^{3}H]Ro 15-4513 (Hall et al. 1992). The addition of clonazepam (1 μM) inhibited the binding of [^{11}C]Ro 15-4513 markedly but incompletely as compared to the inhibition by unlabelled Ro 15-4513 both in the neocortex and in the cerebellum (Fig. 4C).

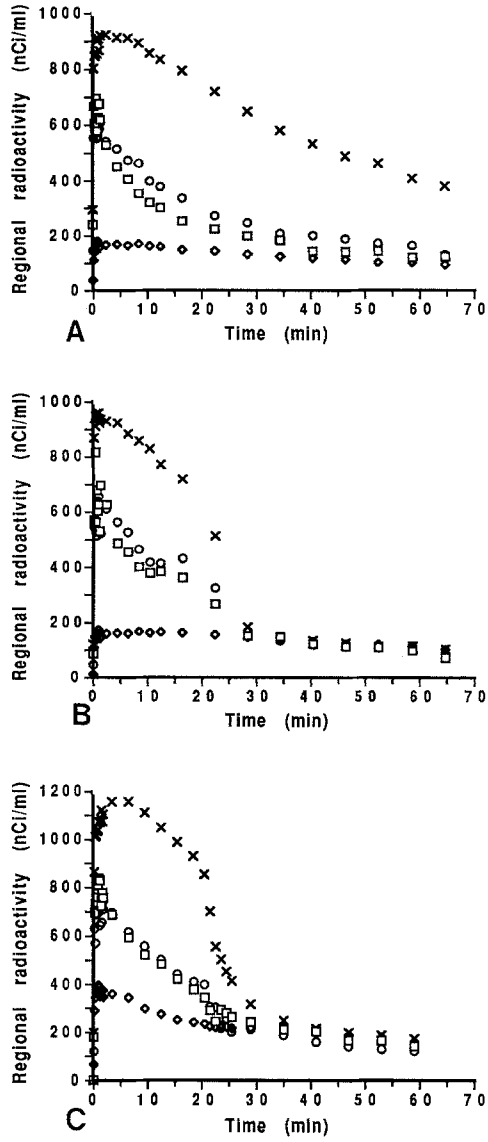

Fig. 5. **A** Time course for regional radioactivity (nCi/ml) in the brain of a Cynomolgus monkey after intravenous administration of 50 MBq [^{11}C]Ro 15-4513. **B** Displacement experiment with 5 mg Ro 15-4513 injected 19–21 min after [^{11}C]Ro 15-4513 (50 MBq). **C** Displacement experiment with 5 mg clonazepam injected 19–21 min after [^{11}C]Ro 15-4513 (75 MBq). (×) Neocortex; (◇) muscle; (○) cerebellum; (□) pons

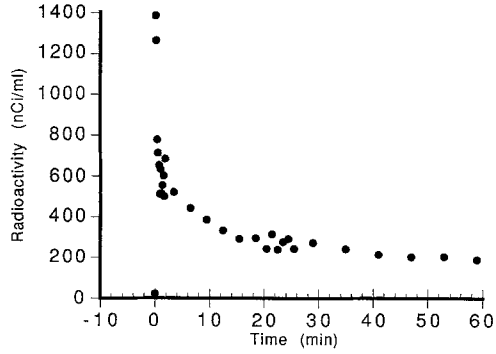

Fig. 6. Time course of radioactivity (nCi/ml) in left and right carotid artery (pooled data) after intravenous administration of 75 MBq [^{11}C]Ro 15-4513 into a Cynomolgus monkey

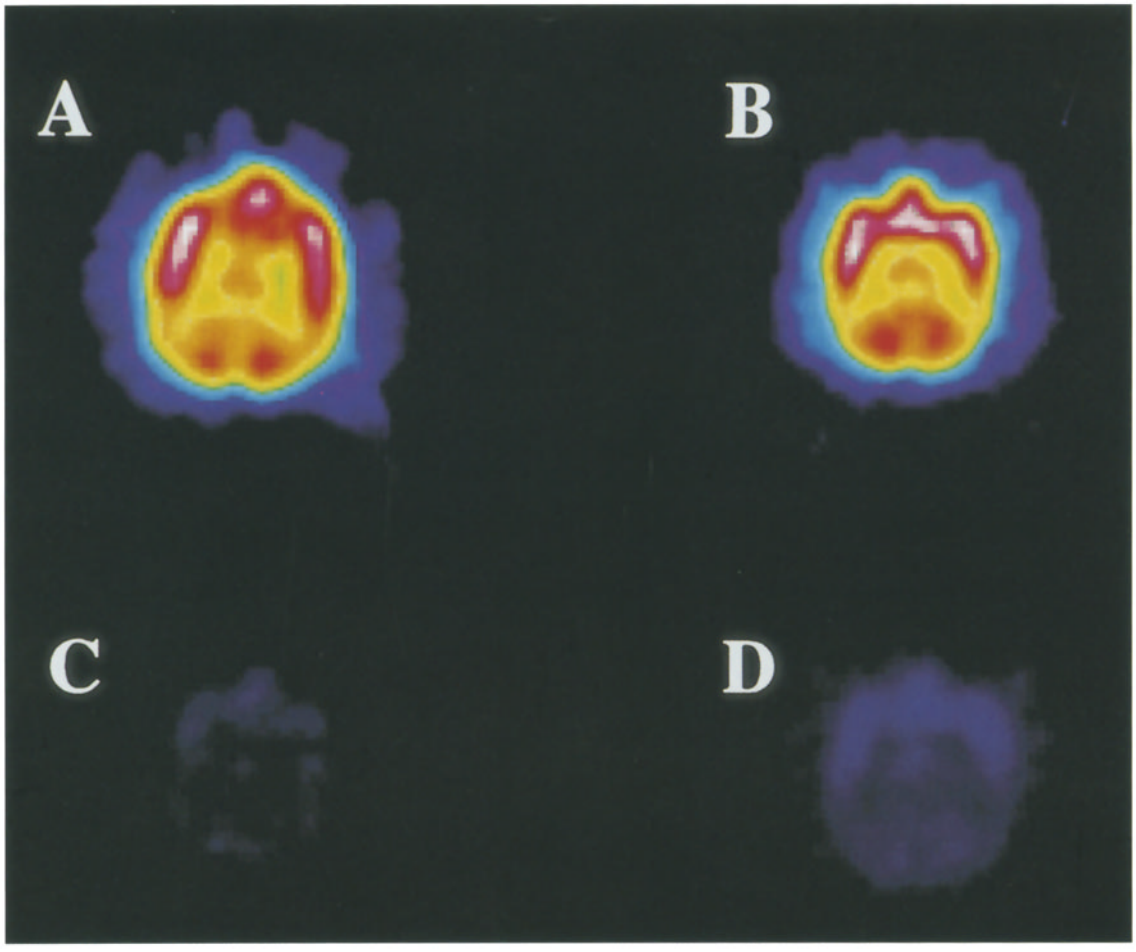

Fig. 7A–D. Colour coded PET images showing the distribution of radioactivity in a horizontal section through the Cynomolgus brain after intravenous administration of (**A**) 50 MBq [^{11}C]Ro 15-4513 (summarized between 0 and 19 min), (**B**) 75 MBq [^{11}C]Ro 15-4513 (summarized between 0 and 19 min), (**C**) same as in (**A**) but after administration of 5 mg Ro 15-4513 between 19 and 21 min after injection of the tracer (summarized between 19 and 65 min), (**D**) same as in (**B**) but after administration of 5 mg clonazepam between 19 and 21 min after injection of the tracer (summarized scans 19–60 min). Frontal is orientated upwards and occipital is downwards on the images

PET experiments

After iv injection of [^{11}C]Ro 15-4513 there was a rapid accumulation of radioactivity in the monkey brain (Figs. 5A and 7A). Two minutes after iv injection 1.2% of the total radioactivity injected was present in the brain. The highest radioactivity was recorded in the neocortex where uptake was maximal already a few minutes after ligand injection. In all brain regions radioactivity was on a higher level than in extracerebral regions as exemplified with the temporal muscle (Fig. 5A).

A high dose (5 mg) of unlabelled Ro 15-4513 (Figs. 5B and Fig. 7C) or clonazepam (Figs. 5C and Fig. 7D) induced a rapid displacement of [^{11}C]Ro 15-4513 binding. This effect was most evident in the neocortex but could also be demonstrated in the pons and the cerebellum. In an extracerebral region corresponding to the temporal muscle there was no effect following injection of Ro 15-4513 or clonazepam.

For the left and right carotid artery a small circular region with a diameter of 4 mm was drawn on PET images corresponding to frames during the time intervals 10–20 and 20–30 s after ligand injection. At this early phase after ligand injection there was high radioactivity in blood and on the PET images. The arteries could be clearly delineated from surrounding tissue. The radioactivity curve for these two regions were pooled. There was a narrow peak within the first 2 min (Fig. 6). The curve was then on a low level during the remaining part of the study. There was no evident effect of any of the displacing compounds on this arterial blood curve.

Discussion

Chemistry

The development of selective ligands labelled with positron-emitting radionuclides, such as ^{11}C or ^{18}F, is essential for the demonstration of receptor binding by PET (Sedvall et al. 1986). Mainly two specific ligands have been prepared for PET examination of BZ receptors:

the antagonist [^{11}C]flumazenil (Ehrin et al. 1984; Maziere et al. 1984; Suzuki et al. 1985; Halldin et al. 1988), and the agonist [^{11}C]suriclone (Frost et al. 1986).

The structural difference between flumazenil and Ro 15-4513 is that the fluorine in the 8-position of flumazenil has been replaced by an azide group (Fig. 1). The preparation and purification of [^{11}C]Ro 15-4513 was thus expected to have several similarities with [^{11}C]flumazenil. In the preparation of [^{11}C]flumazenil two different approaches have been employed: N-methylation of the desmethyl compound with [^{11}C]methyl iodide or esterification of the acid analogue (Ro 15-3890) with [^{11}C]ethyl iodide (Halldin et al. 1988). Both [^{11}C]methyl iodide and [^{11}C]ethyl iodide can routinely be prepared from [^{11}C]carbon dioxide utilizing a one-pot reaction set-up. However, when using longer chain alkyl iodides such as [^{11}C]ethyl iodide, longer reaction time, lower yield and lower specific activity are generally obtained as compared to [^{11}C]methyl iodide. These conditions indicate that N-methylation should be more suitable for routine synthesis of [^{11}C]Ro 15-4513 than esterification with [^{11}C]ethyl iodide.

The radiochemical incorporation of [^{11}C]methyl iodide (Fig. 3) was high (60–70%) and there was no need for further optimization. The purification of [^{11}C]Ro 15-4513 was easily performed by semi-preparative reversed-phase HPLC. HPLC analysis of the final tracer ready for injection was routinely performed using an analytical μ-Bondapak-C18 column. In conclusion, the preparation of [^{11}C]Ro 15-4513 by N-methylation is suitable for PET examination of BZ receptors in the human brain in vivo.

In vitro autoradiography

The autoradiographic studies indicated that [^{11}C]Ro 15-4513 satisfies some essential criteria for a suitable radioligand. Firstly, high density of specific binding sites were found in regions known to be rich with BZ receptors, such as the neocortex and the cerebellum. Secondly, very low accumulation of radioactivity was found in pons and white matter, regions known to have a low density of BZ receptors. Thirdly, virtually all radioactivity was blocked by the addition of a high concentration of unlabelled Ro 15-4513. This indicates that the binding was saturable and that the non-specific binding was low.

High selectivity for one receptor subtype is a criterion for an ideal radioligand which is not satisfied with [^{11}C]Ro 15-4513 as for any of the ligands so far developed for the BZ receptor. [^{11}C]Ro 15-4513 has affinity for BZ receptors on any of the six α-subunits (Lüddens and Wisden 1991). These characteristics were utilized in displacements studies with high concentrations of unlabelled flumazenil or clonazepam (Fig. 4B–C). After displacement the α_6-subunit containing receptors in the cerebellum were selectively visualized. Flumazenil or clonazepam have relatively low affinity for receptors with the α_6-subunit, but for α_1, α_2, α_3 and α_5. Clonazepam was relatively weak in the inhibition of the binding of [^{11}C]Ro 15-4513 in the neocortex (Fig. 4C). The reasons for the relatively weak blockade of neocortical [^{11}C]Ro 15-4513 binding in vitro remains to be examined.

Positron emission tomography

After iv injection of [^{11}C]Ro 15-4513 there was a high and narrow peak of the regional radioactivity curve representing blood in the two carotid arteries (Fig. 6). The shape of this curve was similar to that of arterial blood curves obtained from measurements of radioactivity in arterial blood samples after bolus injection of radioligands (Litton et al. 1987; Eriksson et al. 1988). The use of radioactivity curves from arterial regions in the PET-images may be an experimentally simplified approach as compared to the procedure of obtaining measurements in arterial blood samples. This approach has to be validated in human PET studies.

[^{11}C]Ro 15-4513 passes rapidly across the blood-brain-barrier as indicated by the regional uptake curves for the neocortex, the pons, and the cerebellum (Fig. 5A). Radioactivity was several-fold higher in the cortex than in the pons or the cerebellum. This regional distribution is consistent with the distribution of specific binding demonstrated with autoradiography and indicates that [^{11}C]Ro 15-4513 binds to BZ receptors in vivo. Both unlabelled Ro 15-4513 and the agonist clonazepam markedly displaced brain radioactivity (Figs. 5B–C and 7C–D). The two displacement experiments provided further support for the view that [^{11}C]Ro 15-4513 can be used to visualize specific BZ receptor binding in the primate brain in vivo.

In the autoradiographic study clonazepam did not completely displace [^{11}C]Ro 15-4513 binding in the neocortex and in the cerebellum (Fig. 4C). In the PET study clonazepam induced a very high displacement both in the neocortex and in the cerebellum (Figs. 5C and 7D). The volume of the monkey brain is about 65 ml which is small as compared to the human brain (1300 ml). The resolution of the PET system (4–5 mm FWHM), the small volume of the cerebellum (5 ml), and cerebellum position close to the occipital cortex precludes more definite conclusions regarding displacement in the monkey cerebellum.

In conclusion, the partial inverse BZ agonist [^{11}C]Ro 15-4513 was labelled with a high specific radioactivity. Specific [^{11}C]Ro 15-4513 binding to central BZ receptors was demonstrated both in vitro using autoradiography on cryosections from the human brain and in vivo by PET on Cynomolgus monkeys. On autoradiography, a component of [^{11}C]Ro 15-4513 binding in the cerebellum could not be displaced by flumazenil or clonazepam. This component may reflect binding to the α_6-subunit containing receptors in the granular layers. [^{11}C]Ro 15-4513 should be a useful ligand for PET-examination of BZ receptors in the human brain in vivo.

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