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Comparison of in vivo and ex vivo [^{3}H]flumazenil binding assays to determine occupancy at the benzodiazepine binding site of rat brain $GABA_A$ receptors

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Abstract

In the present study, the occupancy of flumazenil (Ro 15-1788; 1–30 mg/kg p.o.) at the benzodiazepine site of rat brain $GABA_A$ receptors was compared using in vivo and ex vivo binding methodologies with [^{3}H]flumazenil as the radioligand. Animals either received tracer quantities of [^{3}H]flumazenil 3 min before being killed for the in vivo binding, or were killed and brain homogenates incubated with 1.8 nM [^{3}H]flumazenil. The flumazenil dose required to inhibit in vivo binding of [^{3}H]flumazenil by 50% (ID_{50}) was 2.0 mg/kg, which represents the most accurate measure of benzodiazepine site occupancy by flumazenil in vivo. Occupancy measured in crude brain homogenates using the ex vivo method was time dependent with a 3 mg/kg dose giving occupancies of 77% and 12% using 0.5 or 60 min ex vivo incubations times, respectively, presumably due to dissociation from the binding site during the ex vivo incubation. When incubation time was minimised (0.5 min), and despite being under non-equilibrium conditions, the ex vivo method gave an ID_{50} of 1.5 mg/kg which was not too dissimilar from that observed using in vivo binding (2.0 mg/kg). As expected, ex vivo binding can give an underestimation of receptor occupancy but this can be minimised by careful attention to the kinetics of unlabelled drug and radioligand.

Keywords: Flumazenil (Ro 15-1788); In vivo binding; Ex vivo binding; Receptor occupancy; Dissociation; GABA

1. Introduction

The pharmacological effect of any CNS active drug is a function of its intrinsic efficacy as well as the extent to which it binds to its target receptor, enzyme or transporter. Whilst occupancy of the target is related to the pharmacokinetics of the drug, good systemic exposure and penetration through the blood–brain barrier into the CNS do not necessarily result in the availability at the target site in the brain. For example, a compound may be sequestered in a compartment (such as membranes) not accessible to the target such that although pharmacokinetic analyses show that there is compound present in the brain that is unable to interact with its molecular target.

Imaging methods have been developed to determine the extent to which a drug occupies its CNS target, most notably Positron Emission Tomography (PET) and Single Photon Emission Computed Tomography (SPECT) (Smith et al., 2003). Such techniques permit the visualization and quantification of radioligand binding sites in vivo not only in man but also in non-human primates and more recently, with the advent of small animal PET and SPECT scanners, rodents (Beekman and Vastenhouw, 2004; Cherry and Chatiioannou, 2005). The advantage of such imaging methods is that they permit the extrapolation across species using similar methodologies and because of their non-invasive nature allow longitudinal studies to be performed (Phelps, 2000). Moreover, since these methods of measuring receptor occupancy are relatively

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non-invasive they can be used in higher species such as non-human primates, and are safe enough to be employed in man.

From a drug discovery point of view, however, it is often crucial to determine the receptor occupancy in a number of new chemical entities for which in vivo brain imaging studies are not suitable, either in terms of throughput or lack of availability of an appropriate radioligand. For small animal work, the technique of in vivo binding is analogous to PET or SPECT in that the animals receive both unlabelled drug and radiolabel in vivo. However, rather than using radioactive emission detection these experiments rely on animals being killed, the brain was removed and radioactivity was measured using a scintillation counter (Atack et al., 1999, 2005). In order to possess brain penetration and low levels of non-specific binding, ligands need to satisfy a fairly restricted range of physicochemical (i.e. lipophilicity and molecular weight), pharmacological and metabolic criteria (Halldin et al., 2001). With small animals this can be circumvented to a certain extent by the use of ex vivo binding studies, in which drugs are administered to live animals, which are killed, the brain was removed and then homogenised or sectioned and incubated with the non-brain penetrant radioligand, such as a radiolabelled neuropeptide (Duffy et al., 2002; Langlois et al., 2001).

In vivo and ex vivo binding methods clearly have fundamental methodological differences and therefore in the present study we set out to directly compare the occupancy of flumazenil (Ro 15-1788; Hunkeler, 1993) to the benzodiazepine binding site of rat brain $GABA_A$ receptors using in vivo and ex vivo binding techniques and employing [^{3}H]flumazenil as the radioligand. When radiolabelled with tritium, flumazenil makes an ideal in vivo radioligand since it has high affinity for its receptors and is brain penetrant (Witwam and Amrein, 1995). Because of these properties, [^{3}H]flumazenil is an ideal tool for comparing in vivo and ex vivo receptor binding methods. The data clearly show that the occupancy measured by ex vivo binding was dependent upon the incubation time of the assay, with occupancy being greater at shorter incubation times, consistent with flumazenil dissociating from the benzodiazepine site during the ex vivo incubation. However, with short incubation time, the occupancy in the ex vivo assay was comparable to that obtained using in vivo binding.

2. Materials and methods

2.1. Chemicals

Flumazenil and [^{3}H]flumazenil were obtained from Tocris Cookson (Avonmouth, UK) and Perkin–Elmer Life and Analytical Sciences (Boston, MA), respectively. Bretazenil was a gift from Hoffman-La Roche (Basel, Switzerland) and all other chemicals were purchased from Sigma–Aldrich (Gillingham, UK).

2.2. Animals and dosing

All the experiments were performed in accordance with the UK Animals (Scientific Procedures) Act 1986. Male Sprague Dawley rats (200–275 g) were assigned to one of six groups ($n = 9$/group) and received injections (1 mL/kg) of either; vehicle (0.5% methocel p.o.), one of four doses of flumazenil (1, 3, 10, or 30 mg/kg p.o.) or bretazenil (5 mg/kg i.p. in 100% polyethylene glycol), the latter of which occupies all available benzodiazepine binding sites and thereby defines non-specific binding (Atack et al., 1999).

2.3. In vivo binding

Twenty-seven minutes after receiving vehicle, flumazenil or bretazenil, rats received tail-vein injections (1 μL/g) of [^{3}H]flumazenil (Ci/mmol) diluted 1:100 in isotonic saline. Three minutes later, rats were killed by stunning and decapitation, brains were rapidly removed and homogenised in 10 volumes (i.e. 10 mL/g) ice-cold assay buffer (10 mM potassium phosphate buffer plus 100 mM KCl, pH 7.4) using a polytron homogeniser. Three separate aliquots of homogenate (300 μL) were filtered through Whatman GF/B filters and washed with 10 mL ice-cold buffer. The filters were placed in 10 mL liquid scintillant, shaken for at least 1 h and counted on a Beckman LS6500 scintillation counter.

2.4. Ex vivo binding

Thirty minutes after receiving vehicle, flumazenil or bretazenil, rats were killed by stunning and decapitation and brains were rapidly removed, sectioned down the midline and both hemispheres were frozen on dry ice and stored at −70 °C until assayed.

2.4.1. Kinetics of association and dissociation of [^{3}H]flumazenil

On the day of assay, single hemispheres of four vehicle-treated rats were homogenised (1 g in 20 mL) in assay buffer (10 mM potassium phosphate buffer plus 100 mM KCl, pH 7.4) and then 50 μL aliquots were added to 400 μL assay buffer plus 18 nM [^{3}H]flumazenil (final assay concentration = 1.8 nM). In order to measure the rate of association of [^{3}H]flumazenil, incubations were carried out for 0.5–60 min at 4 °C and were terminated by filtration through Whatman GF/B filters and washed with 10 mL of ice-cold assay buffer. Filters were placed in 10 mL of scintillation fluid and counted on a Beckman LS6500 scintillation counter. To study the dissociation rate of flumazenil from the $GABA_A$ receptor benzodiazepine binding site, the same samples used for the association experiments were incubated for 1 h at 4 °C to reach equilibrium, then 10 μM of unlabelled flumazenil was added to prevent [^{3}H]flumazenil rebinding once it had dissociated (i.e. shift the association–dissociation equilibrium in favour of dissociation). Assays were terminated at various times (0.5–60 min) after the addition of flumazenil by filtration and washing as described above. Washed filters were placed in 10 mL of scintillation fluid and counted on a Beckman LS6500 scintillation counter.

2.4.2. Effect of incubation time on occupancy

Hemispheres from vehicle-, flumazenil- (3 mg/kg) and bretazenil-treated animals were thawed, homogenised and the binding of 1.8 nM [^{3}H]flumazenil was assayed as described above using varying incubation times (0.5–60 min). At each time point the non-specific binding in homogenates of bretazenil-treated brains was subtracted from counts in vehicle- or flumazenil-treated brain samples. Occupancy in the 3 mg/kg flumazenil group was defined as the extent to which specific binding was reduced relative to vehicle-treated animals. For example, if specific binding in the vehicle- and flumazenil-treated groups was 6000 and 2000 dpm, respectively, then the occupancy in the latter group would be 67%.

2.4.3. Ex vivo occupancy dose–response curve

Having defined the optimal conditions for determining ex vivo occupancy in the preceding experiments, hemispheres from all dose groups were thawed, homogenised (1:20 weight:volume) and 50 μL aliquots were incubated for 0.5 min in a final assay volume of 0.5 mL containing 1.8 nM [^{3}H]flumazenil. Assays were terminated and processed as described above with non-specific binding being defined by radioactivity in the bretazenil-treated homogenates. Occupancy was calculated as the inhibition of [^{3}H]flumazenil binding in flumazenil-treated animals relative to vehicle-treated samples.