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Characterization of [^{11}C]Tetrabenazine as an *In Vivo* Radioligand for the Vesicular Monoamine Transporter

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[^{11}C]Tetrabenazine ([^{11}C]TBZ) is a new *in vivo* radioligand for positron emission tomographic (PET) imaging of vesicular monoamine transporters. The *in vivo* distribution, metabolism and pharmacological specificity of [^{11}C]TBZ has been determined in rodents. Regional mouse brain retention of [^{11}C]TBZ is highest in brain regions with greatest monoaminergic innervation (striatum, hypothalamus) and can be reduced with ligands for the monoamine vesicular transporter (TBZ, ketanserin) but not haloperidol, a dopamine D_2 receptor antagonist. Chromatographic analysis of rat blood demonstrated rapid metabolism of [^{11}C]TBZ to radiolabeled metabolites (α- and β-[^{11}C]dihydrotetrabenazine) resulting from reduction of the 2-keto group. These metabolites, as well as a third potential metabolite, 9-*O*-desmethylTBZ, have been synthesized in unlabeled form and all three were shown to be capable of greatly reducing *in vivo* accumulation of [^{11}C]TBZ in mouse striatum and hypothalamus. Whole body biodistribution of radioactivity after [^{11}C]TBZ injection was determined in rats, and the data used to calculate the expected human dosimetry from this radiotracer. These studies demonstrated that [^{11}C]TBZ can be safely administered for *in vivo* PET imaging and semi-quantitative determination of vesicular monoamine transporters in living human brain, but quantitative pharmacokinetic modeling of radioactivity distribution will be complicated by the presence of pharmacologically active metabolites.

Introduction

Vesicular monoamine transporters are located exclusively in presynaptic monoaminergic terminals, and along with the neuronal monoamine transporters (distinct for dopamine, norepinephrine and serotonin) and the enzymes involved in monoamine biosynthesis are potential targets for preparation of radioligands for *in vivo* imaging. Vesicular amine transporters and neuronal amine transporters are different proteins that have been recently cloned and sequenced (Giros *et al.*, 1992; Liu *et al.*, 1992) and although they both belong to the 12-transmembrane region transporter superfamily (Uhl, 1992) they are pharmacologically distinct (DaSilva and Kilbourn, 1992; Rostene *et al.*, 1992). Additionally, at present there is no evidence for monoamine specific subtypes of vesicular transporters.

Tetrabenazine (TBZ: Fig. 1) is a specific high affinity (IC_{50} = 3 nM) inhibitor of monoamine uptake into vesicles of presynaptic neurons (Scherman *et al.*, 1988; Henry and Scherman, 1989), which shows more selectivity in central pharmacological action and shorter duration of monoamine depletion as compared to reserpine (Pletscher *et al.*, 1962). [^{3}H]Dihydrotetrabenazine ([^{3}H]TBZOH), a simple reduction product of TBZ, has been developed for use in *in vitro* assays of vesicular monoamine transporters, and for *in vitro* autoradiography (Scherman, 1986; Masuo *et al.*, 1990). Using this radioligand, Scherman and coworkers have been able to demonstrate losses of vesicular transporter binding sites in 6-hydroxydopamine lesioned rats (Masuo *et al.*, 1990), and in post-mortem tissue from Parkinson's disease patients (Scherman *et al.*, 1989).

We have recently prepared a carbon-11 labeled form of TBZ (DaSilva *et al.*, 1993a) and demonstrated specific *in vivo* binding of [^{11}C]TBZ in mouse brain (DaSilva and Kilbourn, 1992). For development of this radiotracer for the *in vivo* study vesicular amine transporters of living human brains using positron emission tomography (PET), it was also necessary to (1) further demonstrate the pharmacologic specificity of this radioligand *in vivo*, (2) calculate the expected human radiation dosimetry

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associated with its *in vivo* use and (3) determine the effects if any of peripheral metabolism of the radiotracer. We report here the *in vivo* characteristics of [^{11}C]TBZ and its potential as a quantitative imaging agent for the study of neurodegenerative diseases.

Materials and Methods

Materials

Tetrabenazine was obtained from Fluka. Ketanserin and haldol were purchased from Sigma Chem. Co. 9-*O*-DesmethylTBZ (OH-TBZ) was prepared as previously described (DaSilva *et al.*, 1993a). α-TBZOH and β-TBZOH were synthesized by modifications of literature methods (DaSilva *et al.*, 1993b). All compounds were prepared for injection in 4% ethanol:isotonic sodium phosphate buffer (pH 4.5).

No-carrier-added, high specific activity (> 1000 Ci/mmol) [^{11}C]tetrabenazine was synthesized as previously described and prepared for injection in sterile phosphate buffer (pH 6.0) (DaSilva *et al.*, 1993a).

Biodistribution studies

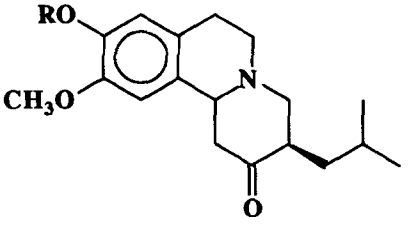
Whole-body biodistribution studies were performed in Sprague–Dawley rats (175–250 g, Charles River). Animals (5 for each data point: 3 females, 2 males) were anesthetized with diethyl ether and [^{11}C]TBZ was injected into the femoral vein at doses of 150–635 μCi of the formulated solution (4 mCi/mL). The animals were allowed to recover, then sacrificed by decapitation at 2, 15, 30 or 60 min after injection. A blood sample was collected and whole tissues were dissected out, assayed for radioactivity (automated γ-counter) and weighed. Radioactivity remaining in the carcass was measured in a dose calibrator. Data were calculated as % injected dose per organ (%ID/organ).

In vivo competition studies

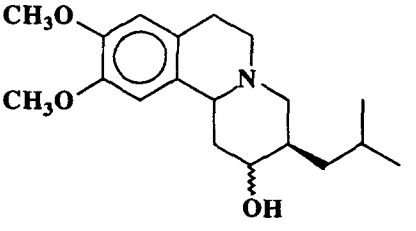
Pharmacological competition studies were done in female CD-1 mice, 20–27 g (Charles River), in a manner similar to that previously reported (DaSilva and Kilbourn, 1992). Experiments were performed with groups of 4 mice per drug dose and an equal number of untreated control animals for each [^{11}C]TBZ formulation used. Competition studies were carried out by either pretreatment of the animals with haloperidol (1 mg/kg, i.p., 30 min prior) followed by i.v. injection of [^{11}C]TBZ, or using i.v. coinjections of test drug [ketanserin (2 mg/kg), α-TBZOH (10 mg/kg), β-TBZOH (10 mg/kg) or OH-TBZ (10 mg/kg)] and [^{11}C]TBZ. For all studies animals were anesthetized (diethyl ether) and i.v. injections were done via the tail vein. The mice were allowed to recover, then sacrificed by decapitation at 10 min after injection. A blood sample was collected, and the brain rapidly removed and dissected into samples of striatum, cortex, hippocampus, hypothalamus, cerebellum and thalamus. All samples were assayed for radioactivity (automated gamma counter) and then weighed. These data were used to calculate the % injected dose per gram (%ID/g) for blood and for each brain region.

Metabolite studies

Metabolite studies were done in a manner analogous to that previously reported (Kilbourn *et al.*, 1989). [^{11}C]TBZ (8.5 mCi in 1.0 mL) was injected via the femoral vein into a Sprague–Dawley rat (200 g). The animal was killed at 15 min and a blood sample (1.0 mL) collected with a heparinized syringe. Ethanol (0.5 mL) was added to half of the blood sample (0.5 mL) to effect hemolysis, then the mixture was vortexed briefly and centrifuged. The supernatant was drawn off and diluted with 9 mL $NaHCO_3$ (7%) ($NaHCO_3$ was used to ensure formation of the free base form of TBZ and its metabolites). The mixture was passed through a C_{18} Sep-Pak (Waters Co.), that was preactivated with ethanol (10 mL) and a solution of 7% $NaHCO_3$ (20 mL). The Sep-Pak was then washed with 10 mL of aqueous $NaHCO_3$ (7%), 10 mL dichloromethane and 10 mL ethanol. These fractions were then assayed for radioactivity (automated γ-counter) and the organic solvents (CH_2Cl_2



R = CH_3 Tetrabenazine (TBZ)
R = H Desmethyltetrabenazine (OH-TBZ)



Dihydrotetrabenazine (TBZOH)

Fig. 1. Structures of tetrabenazine, desmethyltetrabenazine (OH-TBZ) and dihydrotetrabenazine (TBZOH).

Table 1. Calculated cumulative absorbed dose for administration of [^{11}C]TBZ to humans

Organ	rad/mCi
Whole body	0.0077
Red marrow	0.0077
Ovaries	0.0448
Testes	0.102
Brain	0.0558
Heart wall	0.0096
Kidney	0.0317
Liver	0.0389
Lung	0.0062
Spleen	0.011

and ethanol) were evaporated to dryness under reduced pressure on a rotary evaporator. Thin-layer chromatography (TLC) (0.25 mm, glass-backed silica gel plates, 60A K6F, Whatman; eluting solvent mixture: $CHCl_3$/methanol 24/1) was used for establishing the structure of the metabolites. This system clearly separates TBZ ($R_f = 0.62$), OH-TBZ ($R_f = 0.43$), α-TBZOH ($R_f = 0.21$) and β-TBZOH ($R_f = 0.18$). Chromatogram radioactivity was examined with a Berthold linear analyzer. Control experiments were performed with rat blood and authentic [^{11}C]TBZ (>95% purity) to determine recovery capabilities and loss of the radiotracer in the intermediate washings.

Statistical analysis

Statistical analysis was conducted using an unpaired Student's t-test. A $P < 0.05$ was considered statistically significant.

Results

Tissue distribution

After i.v. injection of [^{11}C]TBZ radioactivity levels were highest in the organs involved in metabolism (liver, intestines, kidney) and remained that way throughout the study period (data not shown). High levels of radioactivity were observed in the brain at an early time point (3.37 %ID/organ at 2 min) and then declined rapidly (0.41 %ID/organ at 60 min). A mean of 93% of the total injected dose could be accounted for in the organs and carcass at the four time points studied. The biodistribution data were used for the calculation of expected human dosimetry.

Calculation of human absorbed radiation dose

The expected absorbed doses to humans shown in Table 1 were calculated using the rat biodistribution data from [^{11}C]TBZ injection, following the MIRD formalism (Loevinger and Berman, 1976). The percent administered dose per organ values were modified to reflect the different proportions of organ to total body mass in rat and man (Roedler, 1980). Residence times were obtained by integration under the organ time vs activity curves, with the effective half-life of [^{11}C]TBZ assumed to be equal to the physical half-life of ^{11}C for times exceeding the last data point. Residence times were entered into the MIRDOSE2 program for the generation of absorbed doses to selected target organs per unit administered activity.

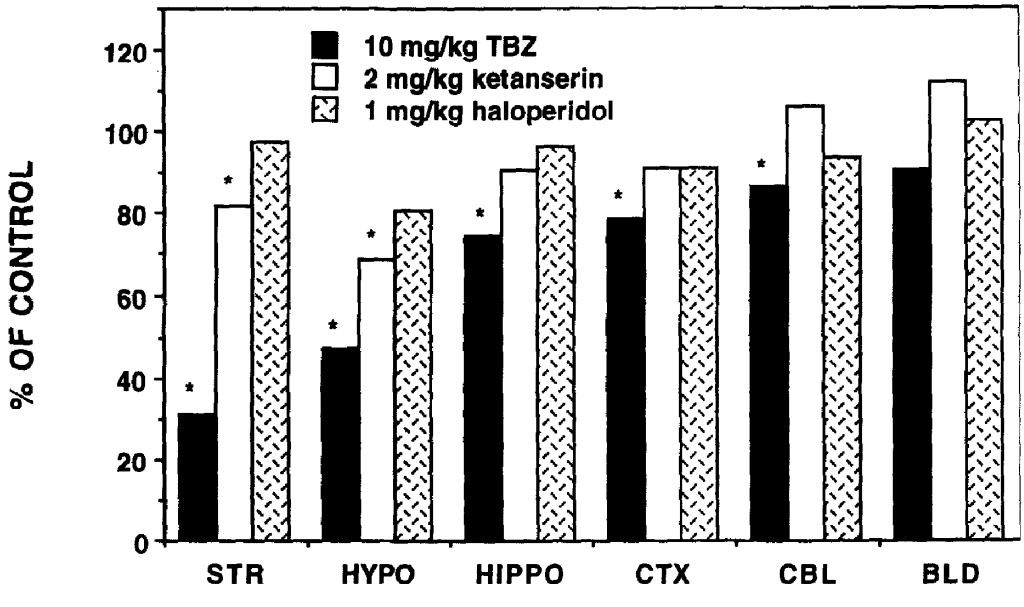


Fig. 2. Effects of coinjections of TBZ or ketanserin, or pretreatment with haloperidol (i.p., 30 min prior), on the mouse brain distribution of radioactivity determined 10 min after i.v. injection of a trace dose of [^{11}C]TBZ. Data are shown as percent of control values (saline pretreatment). Tissues: STR, striatum; HYPO, hypothalamus; HIPPO, hippocampus; CTX, cortex; CBL, cerebellum; BLD, blood. The asterisk (*) indicates $P < 0.05$ compared to controls.

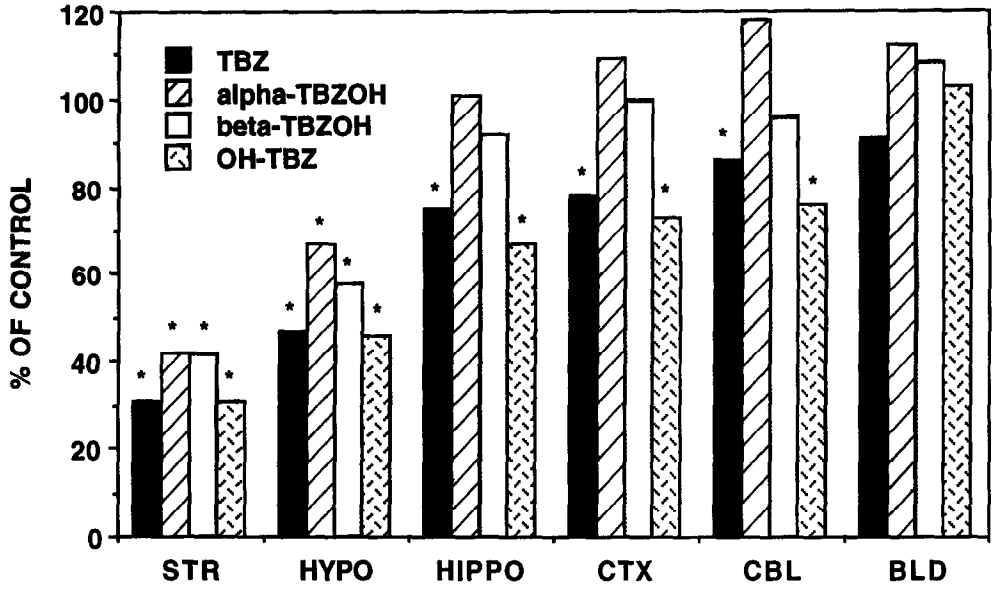


Fig. 3. Effects of coinjections (10 mg/kg of each drug) of TBZ, α- and β-TBZOH and OH-TBZ on the mouse brain distribution of radioactivity determined 10 min after i.v. injection of a trace dose of [^{11}C]TBZ. Data are shown as percent of control values (saline coinjections). Tissues: STR, striatum; HYPO, hypothalamus; HIPPO, hippocampus; CTX, cortex; CBL, cerebellum; BLD, blood. The asterisk (*) indicates $P < 0.05$ compared to controls.

Pharmacological competition experiments

The results from drug competition studies are shown in Figs 2 and 3. Data are presented as % of control animals values determined in the same experimental set; for comparison purposes, values from the coinjection of 10 mg/kg i.v. TBZ are included (DaSilva and Kilbourn, 1992). Statistical significance was determined using the actual data for %ID/g for each brain region. Coinjection with ketanserin (2 mg/kg, i.v.) significantly reduced [^{11}C]TBZ concentrations in the striatum and hypothalamus as compared to controls (Fig. 2); use of higher doses was limited by animal mortality. Pretreatment with 1 mg/kg haldol (i.p., 30 min prior) had no significant effect on [^{11}C]TBZ levels of any brain region or the blood (Fig. 2). The potential metabolites α-TBZOH, β-TBZOH and OH-TBZ all significantly blocked the specific binding of [^{11}C]TBZ in striatum and hypothalamus (Fig. 3). OH-TBZ proved nearly as potent as TBZ itself, and significant reductions in radiotracer accumulation in cortex, hippocampus and cerebellum were also evident.

Metabolism experiments

The distribution of radioactivity from [^{11}C]TBZ and labeled metabolites in rat blood at 15 min following injection of [^{11}C]TBZ is summarized in Table 2. The percentage of total radioactivity that partitioned into plasma upon centrifugation (after ethanol hemolysis) was 85% (15% in the pellet). TLC analyses of the organic eluants from the Sep-Pak separation of the supernatant showed unmetabolized [^{11}C]TBZ (in CH_2Cl_2 fraction) and radioactive metabolites of [^{11}C]TBZ with the same chromatographic mobilities (R_f values) as α-TBZOH and β-TBZOH (present in both CH_2Cl_2 and ethanol fractions). Together, these lipophilic metabolites constituted 88% of the supernatant radioactivity. Hydrophilic metabolites, including potentially any [^{11}C]methanol, were eluted in the $NaHCO_3$ fraction and constituted 8% of the supernatant radioactivity. Finally, a small amount (4%) of material was retained on the Sep-Pak and could not be identified.

Discussion

In vivo imaging of monoaminergic nerve terminals, and in particular dopaminergic nerve terminals, would be of great value for the study of the development and progression (and possible therapeutic treatment or prevention) of neurodegenerative diseases such as Parkinson's disease. The quantitative imaging of vesicular transporters for monoamines, a specific

Table 2. Distribution of radioactivity in the rat blood, 15 min after injection of [^{11}C]TBZ, as determined by C-18 Sep-Pak chromatography

	% of Radioactivity in supernatant*
[^{11}C]TBZ	31
[^{11}C]TBZOH(α + β)	57
Hydrophilic [^{11}C] metabolites	8
C_{18}	4
Total	100

*Supernatant obtained by ethanol hemolysis of blood followed by centrifugation.

presynaptic neuronal function, offers a new approach to this difficult question of quantitation of nerve terminal densities *in vivo*.

Quantitative PET imaging places certain important restrictions on radioligand design and development. First, adequate radiotracer must reach the tissue or region of interest to allow imaging with statistics adequate for pharmacokinetic modeling; unfortunately, for radiotracers destined for brain imaging, most of the radioactive dose does not reach the brain and the absorbed radiation dose to peripheral organs may limit allowable injectable doses for human studies. Second, within the tissue of interest, distribution of the radioactivity should be assignable to interaction with only one binding site. Finally, radioactivity within a region of interest should be attributed to one (or predominantly only one) radioactive species as external imaging cannot distinguish the chemical forms of radioactivity present in the field of view. Thus, formation of radioactive metabolites which have access to the tissue under study can severely complicate quantitative pharmacokinetic modeling of the distribution of the injected radiotracer.

As shown in Table 1, [^{11}C]TBZ meets the first of these requirements. The radioligand showed good rat brain uptake (2.37% injected dose/organ at 2 min) and clearance kinetics similar to those reported previously in mice (DaSilva and Kilbourn, 1992). The low lung uptake (1.11 %ID/organ at 2 min) is appropriate for a radiotracer with relatively moderate lipophilicity [TBZ, log octanol/buffer partition coefficient (log P) = 2.68] (Scherman *et al.*, 1988). Radiation dosimetry is acceptable: the limiting organ for administration of the radiotracer for research purposes would be the testes (3 rad maximum), with an allowable dose of 29 mCi.

The pharmacological specificity of [^{11}C]TBZ distribution *in vivo* is also quite acceptable, as shown in Figs 2 and 3. Radiotracer accumulation can be reduced by treatments with drugs known to act at the vesicular transporter, such as reserpine, tetrabenazine and ketanserin. Ketanserin shows a smaller effect (Fig. 2), perhaps due to the lower affinity of this drug for the vesicular transporter binding site (Darchen *et al.*, 1988) or insufficient concentrations of the drug in the brain after a 2 mg/kg dose; we were prevented from administering higher doses by the toxicity of the drug. Since its initial discovery, TBZ has been considered to have excellent pharmacological specificity both *in vitro* and *in vivo*, with only binding to dopamine D_2 receptors reported as a possible secondary binding site (Reches *et al.*, 1983; Login *et al.*, 1982). Most recently, the *in vivo* binding of the high affinity D_2 receptor antagonist [^{11}C]raclopride was shown to be reduced by administration of a pharmacological dose of TBZ (Dewey *et al.*, 1993). In mouse brain we have found that uptake and retention of radioactivity from [^{11}C]TBZ is unaffected by haloperidol, a dopamine D_2 receptor antagonist (Fig. 2). The data obtained here, in combination with the lack of [^{11}C]TBZ accumulation in the striatum of a MPTP-lesioned monkey (DaSilva *et al.*, 1993a), provides evidence that at a *tracer* level [^{11}C]TBZ predominantly binds to the presynaptic vesicular transporter site. At higher concentrations, such as those obtained from a pharmacological dose, binding to other receptors cannot be ruled out; fortunately, this is not a consideration for *in vivo* imaging of trace doses of radioactive drug.

The metabolism of TBZ has been previously studied in some detail. 9-*O*-DesmethylTBZ and TBZOH (both α- and β-isomers) have been identified as plasma metabolites formed *in vivo* in both animals and man (Schwartz *et al.*, 1966; Mehvar *et al.*, 1986, 1987), and TBZOH has been demonstrated to be present in rat brain following administration of TBZ. From our analysis of rat blood after injection of [^{11}C]TBZ (Table 2), [^{11}C]TBZOH is clearly formed from metabolism of this radioligand. The reduction products, α-TBZOH and β-TBZOH, have been shown *in vitro* to have high affinity for the vesicular monoamine transporter (3 and 20 nM, respectively) (Scherman *et al.*, 1988), and TBZOH shows pharmacokinetics and monoamine-depleting actions similar to that of the parent drug TBZ (Mehvar and Jamali, 1987). This has led to proposals that TBZOH is actually the pharmacologically important species in man following administration of TBZ. As shown in Fig. 3, all three metabolites of TBZ (OH-TBZ, α- and β-TBZOH) are capable of crossing the blood–brain barrier and blocking selective retention of [^{11}C]TBZ in striatum and hypothalamus of mouse brain, with nearly equal efficacy as TBZ. Our data are consistent with the reported pharmacological actions of TBZOH, and confirm that the monoamine-depleting action of that metabolite is due to its affinity for the vesicular transporter. These data also suggest strongly that the distribution of radioactivity after peripheral administration of [^{11}C]TBZ more properly reflects both [^{11}C]TBZ and labeled metabolites, α- and β-[^{11}C]TBZOH. As we have labeled [^{11}C]TBZ in the 9-methoxy group, any OH-TBZ formed by metabolic ether cleavage at that position (Schwartz *et al.*, 1966) would not be radiolabeled, and thus will not contribute to brain radioactivity content.

In studies using monkeys and humans, we have demonstrated that [^{11}C]TBZ can be successfully used to image vesicular amine transporters in the basal ganglia (DaSilva *et al.*, 1993b; Kilbourn *et al.*, 1993). The studies of [^{11}C]TBZ reported here suggest that this radioligand will not be optimal for quantitative pharmacokinetic modeling due to the probable formation of the labeled metabolites α- and β-[^{11}C]TBZOH, which quite likely enter the brain and contribute to the radioactivity distribution. However, as these metabolites are themselves high affinity ligands for the same binding site with similar pharmacokinetics *in vivo*, this radioligand is very suitable for semi-quantitative imaging of vesicular amine trans-

porters and in particular the dopaminergic nerve terminals of the human basal ganglia. Tetrabenazine, rather than a derivative, was chosen as the initial target for radioligand development due to the wealth of data on its pharmacology and toxicology; this allowed for rapid approval of the radiolabeled drug for human studies, and thus the potential human applications of this class of radiotracers could be evaluated. We are now concentrating our efforts on the synthesis and characterization of TBZ derivatives which will not suffer from the formation of radiolabeled metabolites which can enter the brain.

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