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Altered $GABA_A$ receptor density and unaltered blood–brain barrier [^{11}C]flumazenil transport in drug-resistant epilepsy patients with mesial temporal sclerosis

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Abstract

Studies in rodents suggest that flumazenil is a P-glycoprotein substrate at the blood–brain barrier. This study aimed to assess whether [^{11}C]flumazenil is a P-glycoprotein substrate in humans and to what extent increased P-glycoprotein function in epilepsy may confound interpretation of clinical [^{11}C]flumazenil studies used to assess gamma-aminobutyric acid A receptors. Nine drug-resistant patients with epilepsy and mesial temporal sclerosis were scanned twice using [^{11}C]flumazenil before and after partial P-glycoprotein blockade with tariquidar. Volume of distribution, nondisplaceable binding potential, and the ratio of rate constants of [^{11}C]flumazenil transport across the blood–brain barrier (K_1/k_2) were derived for whole brain and several regions. All parameters were compared between pre- and post-tariquidar scans. Regional results were compared between mesial temporal sclerosis and contralateral sides. Tariquidar significantly increased global K_1/k_2 (+23%) and volume of distribution (+10%), but not nondisplaceable binding potential. At the mesial temporal sclerosis side volume of distribution and nondisplaceable binding potential were lower in hippocampus (both ~−19%) and amygdala (both ~−16%), but K_1/k_2 did not differ, suggesting that only regional gamma-aminobutyric acid A receptor density is altered in epilepsy. In conclusion, although [^{11}C]flumazenil appears to be a (weak) P-glycoprotein substrate in humans, this does not seem to affect its role as a tracer for assessing gamma-aminobutyric acid A receptor density.

Keywords

Blood–brain barrier, flumazenil, P-glycoprotein, positron emission tomography, temporal lobe epilepsy

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Introduction

Flumazenil binds to the gamma-aminobutyric acid A ($GABA_A$) receptor, but has no agonistic or antagonistic actions on this receptor. The positron emission tomography (PET) radioligand [^{11}C]flumazenil is used widely for assessing changes in $GABA_A$ receptor density and to assist in determining the site of seizure onset prior to resective surgery in medically refractory epilepsy patients.[1,2] Recent *ex vivo* and *in vivo* data, however, suggest that [^{11}C]flumazenil is a P-gp substrate in rodents.[3–5] It has been hypothesized that P-glycoprotein (P-gp) is upregulated in areas with epileptic activity.[6] If [^{11}C]flumazenil is indeed a P-gp substrate in humans, upregulation of P-gp at the blood–brain barrier (BBB) due to epilepsy could lead to reduced cerebral uptake of [^{11}C]flumazenil, and thus to erroneous interpretation of $GABA_A$ receptor density changes.

The impact of P-gp on cerebral uptake of [^{11}C]flumazenil can be investigated by pharmacological inhibition of P-gp with tariquidar. This compound is one of the most effective P-gp inhibitors, binding to P-gp with high selectivity and affinity.[7–9]

The purpose of the present study was to assess whether [^{11}C]flumazenil is a P-gp substrate in humans and, if so, to what extent changes in cerebral [^{11}C]flumazenil uptake in drug-resistant patients are due to changes in P-gp activity rather than $GABA_A$ receptor density. To address these issues, scans were performed in drug-resistant patients with temporal lobe epilepsy (TLE) and evidence of unilateral mesial temporal sclerosis (MTS) on magnetic resonance imaging (MRI). This syndrome with partial seizures is one of the most prevalent and refractory types of epilepsy with only 20% of patients achieving seizure freedom on medication.[10–12] Seizures often originate in the areas identified on MRI, have a highly characteristic video-electroencephalography (EEG) pattern, both in terms of ictal rhythms and with respect to the ictal semiology.[13] These areas may show stronger increase in P-gp activity than other brain regions and thereby present a suitable target to test the proposed hypothesis.

Materials and methods

Participants

Eleven drug-resistant patients with TLE and unilateral MTS between 18 and 60 years of age were recruited from the outpatient clinics of the tertiary referral centre for epilepsy patients Stichting Epilepsie Instellingen Nederland (SEIN). Diagnosis of TLE and unilateral MTS was based on clinical evaluation, EEG, and MRI. All subjects underwent standard screening, including medical history, physical and neurological examination, screening laboratory tests, and brain MRI to exclude serious medical conditions, psychiatric illness, drug abuse, and coagulation problems. Patients with MRI abnormalities other than those suggesting presence of MTS, white matter changes, or an incidental small lacunar lesion were excluded. Other exclusion criteria were use of benzodiazepines, non-steroidal anti-inflammatory drugs, antithrombotics, acetylsalicylic acid, or drugs known to interfere with P-gp,[14–16] other than antiepileptic drugs. Written informed consent was obtained from each participant. The study was approved by the Medical Ethics Review Committees of VU University Medical Center and SEIN.

MRI

All patients underwent a structural MRI scan using a 3T scanner (Signa HDXt, General Electric, Milwaukee, USA) according to a fixed protocol, including T1-weighted 3D magnetization-prepared rapid acquisition gradient echo (MPRAGE) covering the whole brain, T2-weighted axial fluid-attenuated inversion recovery images, and T2 axial images. The coronal T1-weighted MPRAGE sequence was used for coregistration with the PET scan and for region of interest (ROI) definition.

PET data acquisition

All patients underwent two identical PET scans on the same day. Scans were performed on an ECAT EXACT HR+ scanner[17] (Siemens/CTI, Knoxville, USA), which enables acquisition of 63 transaxial planes of data over a 15.5 cm axial field of view, thus allowing the whole brain to be imaged in a single bed position. To minimize movement artifacts, the head was immobilized and, using laser beams, its position checked for movement during scanning. All patients received an indwelling radial artery cannula for blood sampling and a venous cannula for tracer administration. During the entire PET scanning day, patients were monitored using EEG and video to identify possible ictal events. Subsequently, video-EEG recordings were reviewed by two qualified neurophysiologists with experience in EEG-CCTV seizure monitoring (DNV and JZ). Before tracer injection, a 10-minute transmission scan was performed in 2D acquisition mode using three retractable rotating line sources. This scan was used to correct the subsequent emission scan for photon attenuation. After the transmission scan, a dynamic emission scan in 3D acquisition mode was started simultaneously with an intravenous injection of about 370 MBq [^{11}C]flumazenil by means of an infusion pump (Med-Rad, Beek, the Netherlands; injection rate $0.8\,mL{\cdot}s^{-1}$ followed by a flush of 35 mL saline at $2.0\,mL{\cdot}s^{-1}$). The emission scan consisted of 16 frames

with increasing frame duration (4×15, 4×60, 2×150, 2×300, 4×600 s) with a total duration of 60 minutes. Using an online blood sampler (Veenstra Instruments, Joure, the Netherlands), arterial blood was withdrawn continuously at a rate of $5\,mL \cdot min^{-1}$ for the first 5 min and $2.5\,mL \cdot min^{-1}$, thereafter until 30 minutes after tracer injection. Continuous withdrawal was briefly interrupted at 2.5, 5, 10, 20, and 30 minutes post tracer injection (p.i.) for collection of 10 mL manual blood samples. At 40 and 60 minutes p.i., additional 10 mL blood samples were obtained from the arterial cannula. Blood samples were used to calibrate the online blood sampler curve, and to measure plasma to whole blood radioactivity concentrations and parent [^{11}C]flumazenil fractions in plasma, enabling generation of a metabolite-corrected plasma input curve. The last 30 minutes of the input curve, when there was no continuous blood sampling, were extrapolated using the manual samples collected at 40 and 60 minutes. After a resting period of at least 3 hours to allow for decay of carbon-11, the scanning procedure was repeated, but this time after tariquidar administration at a dose of $2\,mg \cdot kg^{-1}$ body weight administered as a 30 minutes intravenous infusion 110 minutes prior to the [^{11}C]flumazenil scan. For formulation of the tariquidar infusion, vials containing $7.5\,mg \cdot mL^{-1}$ of tariquidar free base in 10 mL of 20/80 ethanol/propylene glycol (v/v) (AzaTrius Pharmaceuticals Pvt Ltd, London, UK) were diluted with aqueous dextrose solution (5%) to yield a final volume of 250 mL. During the post tariquidar [^{11}C]flumazenil PET scan one additional manual blood sample was taken to determine plasma concentration of tariquidar ($T = 5$ minutes p.i.). Subjects were monitored for changes in blood pressure and heart rate during and after tariquidar administration.

PET data analysis

All PET sinograms were corrected for dead time, scatter, randoms, decay, and tissue attenuation, and were reconstructed with a standard filtered back-projection algorithm, using an image matrix size of $256 \times 256 \times 63$, resulting in voxel sizes of $1.2 \times 1.2 \times 2.4$ mm. After reconstruction a transaxial spatial resolution of ~7 mm in the center of the field of view was obtained. Further data processing was performed using noncommercial software packages. The sum of all PET images was coregistered with the MRI image using Vinci software.[18] Thereafter, obtained transformation coefficients were applied to all PET frames, resulting in dynamic PET and MRI scans having the same orientation. ROIs were defined using PVElab, a software program that utilizes a previously validated probability map of 38 delineated grey matter ROIs.[19] In addition, an attempt was made to identify, in each patient, a region of decreased cerebral [^{11}C]flumazenil uptake of at least 0.5 mL by visual inspection of a summed image of the last 30 minutes of each baseline [^{11}C]flumazenil scan. Visual inspection was performed by a nuclear medicine physician (EC) with extensive experience in reading clinical flumazenil scans. Next, exactly the same ROI was defined on the contralateral side.

Template ROI data were analyzed using two complementary methods, a single-tissue compartment (1TC) model, and the simplified reference tissue model (SRTM).[20] Typically, interpretation of the volume of distribution (V_T) as an index of receptor-binding density assumes that differences in V_T estimates ($V_{ND} + V_S$) are due to differences in specific binding (V_S) and not to those in the nondisplaceable volume of distribution ($V_{ND} = K_1/k_2$). Similarly, estimates of the nondisplaceable binding potential (BP_{ND}) from SRTM assume that K_1/k_2 (V_{ND}) is constant across the brain. By combining both analyses, it is possible to look for changes in the ratio of the rate constants for [^{11}C]flumazenil transport across the BBB (K_1/k_2), and hence [^{11}C]flumazenil efflux (k_2) across the BBB, with sufficient estimation to enable investigation of potential P-gp effects. For this purpose, first, V_T and the rate constant K_1 were obtained using a 1TC model with metabolite-corrected plasma input function and blood volume as a fitting parameter.[20] V_T represents the ratio of flumazenil concentrations in tissue and (metabolite-corrected) plasma under equilibrium conditions. Next, BP_{ND} was determined using the SRTM with pons as reference tissue.[20] To this end, the pons was manually segmented on the MRI image and then projected onto the coregistered dynamic PET data. In order to reduce variability, the pons was delineated once and used for both (coregistered) PET scans. BP_{ND} represents the ratio of $GABA_A$ receptor association over dissociation constants (k_3/k_4) of flumazenil. In terms of a two-tissue compartment (2TC) model, V_T corresponds to $K_1/k_2 \cdot (1 + BP_{ND})$. Consequently, the ratio of the rate constants for [^{11}C]flumazenil transport across the BBB (K_1/k_2) was calculated as $V_T/(1 + BP_{ND})$. Finally, to explore whether tariquidar has an effect on [^{11}C]flumazenil influx (K_1) or efflux (k_2) across the BBB, k_2 was calculated as K_1 divided by K_1/k_2. As the approach mentioned above is an indirect way to obtain K_1/k_2, data were also analyzed using a reversible 2TC model.[20]

For each patient both ROIs were projected onto the dynamic PET scans and values of BP_{ND}, V_T, K_1/k_2, K_1, and k_2 were obtained as described above. Regional analyses were performed with all regions in the hemisphere where evidence of unilateral MTS on MRI was found at one side. This side was called ipsilateral, and all corresponding regions in the other hemisphere were named contralateral.

Statistical analysis

First, in order to test whether [^{11}C]flumazenil is a P-gp substrate, differences in whole brain values of V_T, BP_{ND}, K_1/k_2, K_1, and k_2 between pre- and post-tariquidar scans were evaluated using nonparametric Wilcoxon signed-rank tests. For further exploration, regional values of the significant parameters of the whole brain analyses as well as pons V_T, K_1, and k_2 (1TC model) were compared between pre- and post-tariquidar scans. Second, baseline regional values of V_T, BP_{ND}, and K_1/k_2 from standard ROIs at the side of MTS were compared to corresponding contralateral ROIs. Third, baseline values of V_T, BP_{ND}, and K_1/k_2 from the manually defined epileptic foci were compared with corresponding regions on the contralateral side. $p < 0.05$ was considered significant.

Spearman's rank-order correlation test was used to assess a correlation between tariquidar plasma concentration levels and V_T, K_1/k_2, and k_2 changes in response to tariquidar. Finally, the nonparametric Wilcoxon signed-rank test was used to assess whether there were significant differences between baseline and posttariquidar values of injected dose, specific activity, fraction of radio-labeled plasma metabolites, and plasma radioactivity concentrations. For all tests $p < 0.05$ was considered significant, except for the latter 2 for which Bonferroni correction for multiple (i.e., 7) comparisons was necessary ($p < 0.007$ was considered significant). Data are presented as mean ± standard deviation (SD) unless stated otherwise.

Results

[^{11}C]flumazenil scans were performed in 11 patients with TLE and unilateral MTS. Due to technical problems with the online blood sampler in one patient and occlusion of the arterial line in another, scans from nine patients could be analyzed. Table 1 shows patient and scan characteristics. Three patients experienced events on the scanning day. One patient (subject 1) experienced a complex partial seizure approximately 90 minutes after tariquidar administration, which was documented on the video-EEG registration. The second patient (subject 3) was temporarily unresponsive, but it was not clear whether this was caused by epileptic activity, as no electrographical abnormalities were observed at that time. Afterwards she was disorientated for a short while and experienced a headache. The third patient (subject 4) became nauseous near the end of the second PET scan, approximately 150 minutes after tariquidar administration. Video-EEG monitoring during the day revealed no seizure activity in any of the patients, except for subject 1. There were no differences between baseline and posttariquidar scans with respect to injected dose (364 ± 33 and 366 ± 30 MBq, respectively; $p = 0.91$), specific activity (131 ± 76 and 162 ± 37 GBq μmol^{-1}, respectively; $p = 0.27$) of [^{11}C]flumazenil. Furthermore, tariquidar had no effect on levels of tracer metabolism and not on plasma activity concentrations. Tariquidar plasma concentrations 5 minutes after [^{11}C]flumazenil injection were on average 278 μg·L^{-1} (range 156–550 μg·L^{-1}). No correlation was found between tariquidar plasma concentration levels and changes in V_T, K_1/k_2, and k_2 in response to tariquidar.

Whole brain analyses using the combination of a 1TC model and SRTM showed that K_1/k_2, V_T, and k_2 significantly altered by 23, 10, and −15%, respectively after tariquidar ($p = 0.008$, $p = 0.012$, and

Table 1. Patient and scan characteristics.

Subject	Gender (M/F)	Age (years)	MRI MTS (L/R)	EEG	Age at onset of epilepsy (years)	Interval last seizure to PET (days)	Duration of epilepsy (years)	Average seizure frequency (per month)	Current AEDs
1	F	31	L	LT	24	2	7	3	CBZ, LEV
2	M	57	L	LT	22	6	35	2	CBZ, PHB
3	F	28	R	RT	8	350[b]	20	1/12[c]	CBZ
4	F	41	R	RFT	39	0.3	2	4	LEV
5	F	29	R	RT	5	10	24	2	LTG, LEV
6	F	58	L	LT	1	86	57	1	CBZ, GBP
7	M	60	L	LFT	9	3	51	6[d]	LTG, LEV
8	F	37	R	RT	17[a]	10	20	3	CBZ, LEV
9	M	33	R	RT	2	6	31	4	CBZ, PHT, VPA

M/F: male/female; MRI: magnetic resonance imaging; MTS: mesial temporal sclerosis; L/R: left/right; EEG: electroencephalography; PET: positron emission tomography; AEDs: antiepileptic drugs; LT: temporal left; RT: temporal right; FT: frontotemporal; CBZ: carbamazepine; LEV: levetiracetam; PHB: phenobarbital; LTG: lamotrigin; GBP: gabapentin; VPA: valproic acid; PHT: phenytoin. [a]Possible unrecognized seizures during childhood. [b]Unreliable due to seizure-related amnesia. [c]Seizure frequency was suspected to be much higher. [d]On average 2 days a month 3 seizures a day.

$p = 0.008$, respectively; Table 2). Whole brain values of BP_{ND} and K_1 were not significantly different between pre- and post-tariquidar scans (both $p = 0.20$; Table 2). With respect to K_1/k_2, V_T, and k_2 responses to P-gp inhibition, no differences were observed between ipsilateral and contralateral hemispheres, not even at a regional level. Pons V_T significantly increased by 17% after tariquidar ($p = 0.011$; Table 3), and a trend was found for a decrease in k_2 of 10.8% after tariquidar ($p = 0.051$). Pons K_1 did not significantly differ between pre- and post-tariquidar scans ($p = 0.57$; Table 3).

Using the same models for assessment of baseline differences between the ipsi- and contralateral ROIs, regional analyses revealed significantly lower V_T and BP_{ND} in ipsilateral hippocampus (−18%, $p = 0.008$ and −20%, $p = 0.008$, respectively), amygdala (−15%, $p = 0.012$ and −17%, $p = 0.012$, respectively), and medial inferior temporal gyrus (−4%, $p = 0.020$ and

Table 2. Whole brain V_T, BP_{ND}, K_1/k_2, K_1, and k_2 of [^{11}C]flumazenil before and after tariquidar administration derived using the 1TC model and SRTM.

	V_T			BP_{ND}			K_1/k_2			K_1			k_2		
Patient	Baseline	Post TQD	Change (%)[a]	Baseline	Post TQD	Change (%)[a]	Baseline	Post TQD	Change (%)[a]	Baseline	Post TQD	Change (%)[a]	Baseline	Post TQD	Change (%)[a]
1	6.47	6.02	−7	7.28	4.21	−42	0.78	1.16	48	0.39	0.41	5	0.50	0.36	−29
2	4.84	5.57	15	4.48	3.91	−13	0.88	1.13	28	0.26	0.27	2	0.30	0.24	−20
3	4.43	5.06	14	4.17	3.90	−6	0.86	1.03	21	0.33	0.38	13	0.39	0.36	−7
4	4.66	5.15	10	4.30	3.97	−8	0.88	1.03	18	0.28	0.31	11	0.32	0.30	−5
5	4.90	5.42	11	4.88	4.13	−15	0.83	1.06	27	0.33	0.33	−1	0.40	0.31	−22
6	5.59	5.72	2	5.28	3.33	−37	0.89	1.32	48	0.37	0.35	−7	0.42	0.26	−37
7	4.87	5.60	15	4.38	4.78	9	0.91	0.97	7	0.26	0.27	7	0.28	0.28	0
8	6.12	7.05	15	3.86	4.65	20	1.26	1.25	−1	0.38	0.37	−3	0.30	0.29	−2
9	4.84	5.33	10	3.51	3.32	−5	1.07	1.23	15	0.29	0.29	−1	0.27	0.23	−14
AVG	5.19	5.66	10	4.68	4.02	−11	0.93	1.13	23	0.32	0.33	3	0.35	0.29	−15
SD	0.70	0.60	7	1.10	0.50	20	0.15	0.12	17	0.05	0.05	6	0.08	0.05	13

V_T: distribution volume (1TC model); BP_{ND}: binding potential (SRTM model); K_1/k_2: ratio of rate constants of [^{11}C]flumazenil transport across the blood–brain barrier (both 1TC model and SRTM); K_1: influx rate constant ($mL \cdot cm^{-3} \cdot min^{-1}$) (1TC model); k_2: efflux rate constant (min^{-1}) (both 1TC model and SRTM); 1TC model: 1 tissue compartment model; SRTM: simplified reference tissue model; TQD: tariquidar; AVG: average; SD: standard deviation. [a]Percentage change after tariquidar administration relative to baseline.

Table 3. Pons V_T, K_1, and k_2 of [^{11}C]flumazenil before and after tariquidar administration derived using the 1TC model.

	V_T			K_1 ($mL \cdot cm^{-3} \cdot min^{-1}$)			k_2 (min^{-1})		
Patient	Baseline	Post TQD	Change (%)[a]	Baseline	Post TQD	Change (%)[a]	Baseline	Post TQD	Change (%)[a]
1	0.87	1.01	17	0.31	0.38	21	0.36	0.38	4
2	0.86	0.97	13	0.25	0.27	7	0.29	0.28	−5
3	0.77	0.91	18	0.27	0.33	22	0.35	0.36	3
4	0.82	1.03	25	0.26	0.30	14	0.32	0.29	−9
5	0.80	0.97	22	0.32	0.31	−4	0.40	0.32	−21
6	0.95	1.17	23	0.39	0.33	−15	0.41	0.28	−31
7	0.79	0.92	15	0.21	0.22	5	0.27	0.25	−9
8	1.10	1.08	−2	0.37	0.35	−7	0.34	0.32	−5
9	0.95	1.14	20	0.28	0.26	−7	0.30	0.23	−23
AVG	0.88	1.02	17	0.30	0.31	4	0.34	0.30	−11
SD	0.10	0.09	8	0.06	0.05	13	0.05	0.05	12

V_T: distribution volume; K_1: influx rate constant ($mL \cdot cm^{-3} \cdot min^{-1}$); k_2: efflux rate constant (min^{-1}); 1TC model: 1 tissue compartment model, TQD: tariquidar; AVG: average; SD: standard deviation. [a]Percentage change after tariquidar administration relative to baseline.

−5%, $p = 0.020$, respectively) as compared with the contralateral side. In all other ROIs no significant left-right differences were observed. In addition, significantly lower K_1/k_2 ratios in ipsilateral hippocampus ($p = 0.008$), superior temporal gyrus ($p = 0.039$) and thalamus ($p = 0.039$) were found, as compared with the contralateral side, although these differences were not substantial (namely between−0.4 and −2%). In only three patients a region of decreased cerebral [^{11}C]flumazenil uptake could be identified by visual inspection. V_T and BP_{ND} were significantly lower in these regions than in the corresponding contralateral ROIs, whereas no differences in K_1/k_2 between regions were observed (Table 4).

In the reanalysis using the 2TC model, one patient (subject 4) had to be excluded because only 40 minutes of the posttariquidar scan data were available, resulting in unreliable fits. In the remaining eight patients, whole brain V_T was 5.69 ± 0.71 and 5.96 ± 0.70 for pre- and post-tariquidar scans, respectively. These values were comparable with those obtained using the 1TC model, but for the 2TC model the difference in V_T between pre- and post-tariquidar scans was not significant ($p = 0.20$). It was not possible to fit regional data reliably using the 2TC model, as too many results had to be rejected because of nonphysiological parameter estimates with high standard errors.

Discussion

The main finding of this study was an increase of 23% in the K_1/k_2 ratio of [^{11}C]flumazenil after partial P-gp blockade. As these rate constants are related to transport of [^{11}C]flumazenil across the BBB, this finding supports the notion that [^{11}C]flumazenil is indeed a P-gp substrate, as demonstrated previously in *in vivo* studies in rodents using both a genetic disruption model and the same pharmacological inhibition model.[3,21] It is also in line with results from an *ex vivo* study in mice.[4] On the other hand, the comparison between assumed site of seizure onset and contralateral side did not show differences in K_1/k_2, and therefore does not provide evidence that P-gp activity is altered at the site of seizure onset in TLE patients. Interestingly, regional analyses showed substantially lower V_T and BP_{ND} in hippocampus and amygdala at the ipsilateral side, but no corresponding change in K_1/k_2. This suggests that the reduction in [^{11}C]flumazenil uptake exclusively reflects a decrease in $GABA_A$ receptor density due to epileptic activity in these regions.

In theory, the increase in K_1/k_2 after partial P-gp blockade could be due to either increased influx (K_1) of [^{11}C]flumazenil from the circulation into the brain or decreased efflux (k_2) of this tracer from the brain to the blood, or both. If [^{11}C]flumazenil is a substrate of P-gp, administration of tariquidar, which results in P-gp inhibition, should lead to decreased efflux (k_2) rather than influx (K_1).[21] The present study confirmed that k_2 rather than K_1 was affected by tariquidar. The finding that V_T was significantly affected by tariquidar, though to a lesser extent than K_1/k_2, is also in line with previous findings, as the brain-to-plasma ratio is expected to increase due to tariquidar. In addition, as pons is almost devoid of $GABA_A$ receptors, the significant increase of 17% in pons V_T (1TC model) after P-gp inhibition also suggests that [^{11}C]flumazenil is a P-gp substrate. Altogether, these results are in line with the notion that flumazenil is a P-gp substrate in humans. In addition, BP_{ND} was not affected significantly by P-gp inhibition, which is also in line with animal studies showing that tariquidar had no effect on [^{11}C]flumazenil binding to the $GABA_A$ receptor in both naïve and kainate-treated rats.[21]

Previous *in vitro* transport assay studies have reported that flumazenil is not transported by human P-gp,[22,23] which is in contrast with findings of previous *in vivo* rodent studies and the present study. These differences have been attributed to species differences in BBB transport of [^{11}C]flumazenil, a phenomenon that has also been observed for other PET radioligands.[24] It is more likely, however, that these contradicting results are due to the lower sensitivity of *in vitro* assays for detecting weak to moderate P-gp substrates.[22]

Table 4. V_T, BP_{ND}, and K_1/k_2 of [^{11}C]flumazenil at ipsilateral and contralateral sides with respect to site of seizure onset.

	V_T			BP_{ND}			K_1/k_2		
Patient	Ipsi	Contra	Difference (%)[a]	Ipsi	Contra	Difference (%)[a]	Ipsi	Contra	Difference (%)[a]
1	3.57	4.70	−24	4.33	5.81	−34	0.67	0.69	−3
6	4.56	5.38	−15	4.39	5.36	−22	0.85	0.85	0
8	4.39	6.07	−28	2.90	4.24	−47	1.13	1.16	−3

V_T: distribution volume; BP_{ND}: binding potential; K_1/k_2: which represents the ratio of the rate constants of [^{11}C]flumazenil transport by P-glycoprotein across the blood–brain barrier; Ipsi: region ipsilateral to the site of seizure onset; Contra: region contralateral to the site of seizure onset.

There may be several reasons for the difference in degree of inhibition of flumazenil transport across the BBB after P-gp blockade with tariquidar in humans (23%) than in rodents (~70%). First, in the *in vivo* rodent studies full P-gp blockade was obtained,[3] whereas this was not possible in the present study in humans,[25] as it was considered unsafe to administer higher tariquidar doses than $2\,mg{\cdot}kg^{-1}$ body weight[25] to patients who also use antiepileptic drugs. Second, species differences in BBB transport of several P-gp substrates have shown a more pronounced increase in cerebral uptake of these substrates after P-gp inhibition in rats than in higher species.[24]

The finding that $GABA_A$ receptor density is focally altered due to epilepsy is in line with earlier studies.[5,21,26] However, the fact that no evidence for locally altered P-gp function was found needs further consideration. Perhaps flumazenil is too weak a P-gp substrate to detect regional alterations in P-gp function. Previous studies on resected brain tissue of refractory epilepsy patients, animal studies and an *in vivo* PET study with the P-gp substrate tracer (*R*)-[^{11}C]verapamil in humans have shown that both P-gp overexpression and P-gp upregulation play a role in drug resistance in epilepsy.[6,27] However, direct clinical proof for P-gp upregulation is scarce. Therefore, more human PET studies with both P-gp substrate and P-gp inhibitor tracers are needed to provide further insight into the presence and, if so, clinical relevance of altered P-gp functionality and expression in drug resistance in epilepsy. As [^{11}C]flumazenil is less affected by P-gp than substrate tracers such as (*R*)-[^{11}C]verapamil and [^{11}C]*N*-desmethyl-loperamide,[25,28] presence and severity of P-gp upregulation can better be assessed using one of those tracers.

The principle analysis used in the current study was based on the combined use of 1TC and SRTM models. Ideally, results should be derived from a single model and, in theory, all kinetic parameters can be obtained using a reversible 2TC model. Unfortunately, the latter model did not provide reliable estimates of individual rate constants and BP_{ND}, which is in line with a previous study showing that distinguishing the two compartments from each other is quite difficult, especially for higher noise levels (small ROIs).[29] Nevertheless, the similarity of whole brain V_T values derived from 1TC and 2TC models, which is in agreement with previous studies,[20,29] indicates that possible bias by lumping the two compartments together in the 1TC model is small. Interestingly, the difference in whole brain V_T between pre- and post-tariquidar scans was significant for the 1TC, but not for the 2TC model. This probably also is due to increasing uncertainty in parameter estimates with increasing number of parameters.

The finding that no correlation was found between tariquidar plasma concentration levels and changes in V_T, K_1/k_2, and k_2 in response to tariquidar probably is due to the fact that flumazenil is only a weak P-gp substrate.

One of the limitations of the present study was the relatively small sample size. In addition, decreased focal cerebral [^{11}C]flumazenil uptake of at least 0.5 mL could be observed with certainty in only one-third of the patients. Therefore, further studies are needed to assess whether there really is no (effect due to) altered P-gp activity at the site of seizure onset. In addition, although tariquidar has been developed as a potent P-gp inhibitor, recently it has been shown that it also inhibits breast cancer resistance protein (BCRP),[7,30] which is another important efflux transporter at the BBB. On the other hand, BCRP inhibition is thought to occur only with pharmacological doses, which are much higher than the tariquidar dose of $2\,mg{\cdot}kg^{-1}$ body weight administered in the present study.[31] Therefore, it is unlikely that BCRP inhibition played a role in the present study. Finally, full P-gp blockade at the BBB could not be obtained because of safety issues. Nevertheless, even partial P-gp blockade indicated that [^{11}C]flumazenil is a P-gp substrate.

In conclusion, this study provides evidence that [^{11}C]flumazenil is a (weak) P-gp substrate in humans. Most importantly, although a P-gp substrate, this does not appear to affect its clinical use as a tracer of $GABA_A$ receptors for localizing the site of seizure onset.

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Authors' contributions

F.E. Froklage contributed to conception and design, acquiring all data, analyzing and interpreting data, drafting and critically contributing to the manuscript, and approving the final content of the manuscript. A. Postnov and M.M. Yaqub contributed to analyzing and interpreting PET data, drafting

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