The Anxiolytic Etifoxine Binds to TSPO Ro5-4864 Binding Site with Long Residence Time Showing a High Neurosteroidogenic Activity

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ABSTRACT: The low binding affinity of the approved anxiolytic drug etifoxine (Stresam) at the steroidogenic 18 kDa translocator protein (TSPO) has questioned the specific contribution of this protein in mediating the etifoxine neurosteroidogenic efficacy. Residence time (RT) at the binding site of the classical TSPO ligand PK11195 is emerging as a relevant neurosteroidogenic efficacy measure rather than the binding affinity. Here etifoxine was evaluated for (i) the in vitro neurosteroidogenic activity in comparison to poorly neurosteroidogenic reference TSPO ligands (PK11195 and Ro5-4864) and (ii) the affinity and RT at [3H]PK11195 and [3H]Ro5-4864 binding sites in rat kidney membranes. Etifoxine shows (i) high neurosteroidogenic efficacy and (ii) low affinity/short RT at the [3H]PK11195 site and low affinity/long RT at the [3H]Ro5-4864 site, at which etifoxine competitively bound. These findings suggest that the long RT of etifoxine at the Ro5-4864 binding site could account for its high neurosteroidogenic efficacy.

KEYWORDS: Etifoxine, residence time, neurosteroidogenic efficacy, anxiolytic effect, kinetic binding parameters

Etifoxine (Stresam Biocodex, Gentilly, France) is a benzoxazine derivative approved for the treatment of anxiety since 1979,[1] and it is currently under investigation for its ability to promote peripheral axonal regeneration[3] and treat pain pathologies.[4] Etifoxine has the important clinical advantage of exerting anxiolytic effects without any adverse effects typical of benzodiazepines (BZDs), such as dependence, anterograde amnesia, sedation, and impaired psychomotor performance.[2,5] Anxiolytic effects of etifoxine, as well as those induced by BZDs, have been attributed to the potentiation of GABAergic transmission by a positive allosteric modulation of type A GABA ($GABA_A$) receptor/chloride ionophore.[6] Analogously to BZDs, etifoxine modulates $GABA_A$ receptor through direct interaction with a specific site that is close to the chloride channel and distinct from that of BDZs.[7] However, an additional indirect mechanism in the modulation of $GABA_A$ receptor by etifoxine was proposed, as this molecule is able to stimulate the endogenous synthesis of 3α-reduced neurosteroids,[8,9] the most potent positive allosteric modulators of $GABA_A$ receptor activity.[10] Etifoxine-mediated neurosteroidogenesis stimulation was suggested to occur by its binding to a mitochondrial protein, the 18 kDa translocator protein (TSPO).[8] TSPO plays a key role in the neuroactive steroid biosynthesis, as converging data have suggested that it is involved in the first rate-limiting step of steroidogenesis.[11] Specifically, TSPO supplies the steroidogenesis substrate cholesterol to the cytochrome P450 enzyme CYP11A1, which converts it into pregnenolone, the precursor of all neurosteroids.[11] Actually, etifoxine efficaciously stimulates neurosteroidogenesis in nervous system,[8,9] mimicking TSPO selective steroidogenic ligands, such as, for example, the anxiolytic candidate XBD173,[12] that, like etifoxine, has been demonstrated to exert antianxiety effects in rodents and humans without the typical undesirable side-effects of BDZs (ClinicalTrials.gov identifier: NCT00108836).[13] Despite these findings, several literature data, mainly those pinpointing the low binding affinity of etifoxine to TSPO,[6,8] have questioned the specific involvement of this protein in the etifoxine pharmacological effects.[14]

Our recent studies have demonstrated that the neurosteroidogenic ability of a TSPO ligand can be related to its "residence time" (RT, which refers to the period a ligand is bound to its target), rather than its binding affinity.[15,16] In particular, an experimental protocol was set up to determine the RT of classical TSPO ligands, such as PK11195 and Ro5-4864, as well as XBD173 and compounds belonging to phenylindolylglyoxylamide class (PIGAs).[17−20] Results highlighted that a long RT at TSPO was necessary so that a TSPO ligand promotes efficaciously neurosteroidogenesis, irrespective of its ligand binding affinity.[15,16]

The aim of the present paper is to explore whether RT might represent the crucial parameter able to rationalize the high neurosteroidogenic activity of etifoxine. In this view, the kinetic binding parameters of etifoxine were assessed for the first time by applying the "competition kinetic association" experimental approach, that follows the theoretical model developed by Motulsky and Mahan.[21] Specifically, the method involves the simultaneous addition of a radioligand for a given biological

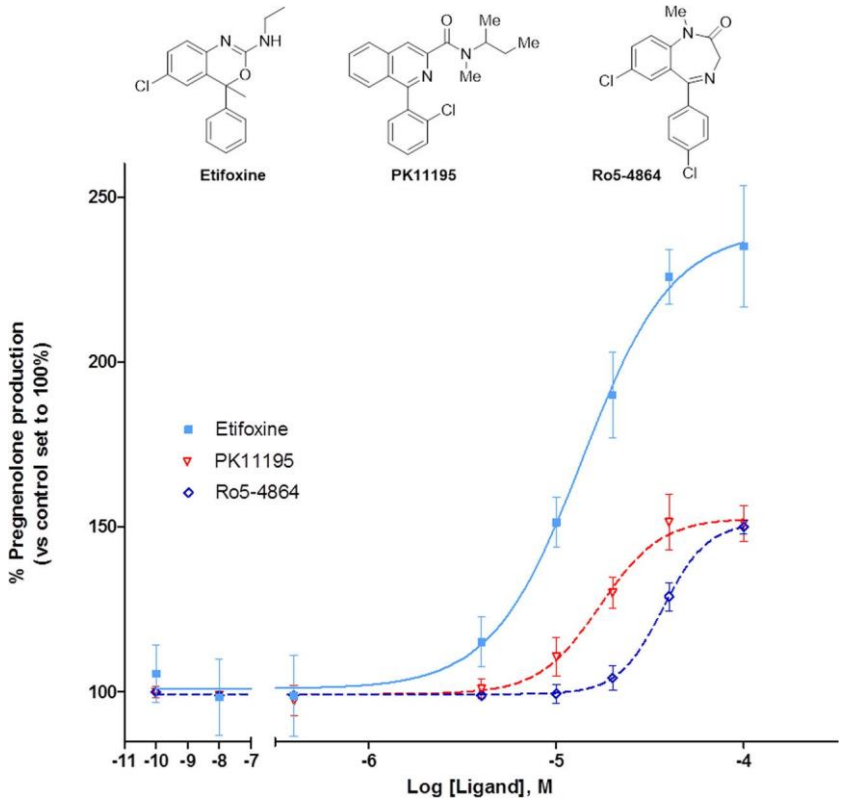

Figure 1. In vitro neurosteroidogenic efficacy of etifoxine. Pregnenolone released from 2 h etifoxine-treated C6 glioma cells was quantified by ELISA. For such a measurement, C6 cells were exposed with increasing concentration of etifoxine in serum-free conditions in the presence of trilostane and SU10603, specific inhibitors of further pregnenolone metabolism. The graph reports also data obtained by the use of the TSPO ligands PK11195 and Ro5-4864 with low steroidogenic activity. The results are expressed as the mean ± SEM of three separate experiments. The efficacy values of etifoxine, PK11195, and Ro5-4864 (calculated at 100 μM) were 235 ± 18%, 151 ± 5% and 150 ± 2%, respectively (vs control, set up at 100%). Chemical structures of etifoxine, PK11195, and Ro5-4864 are also shown.

target and a competitor to the receptor preparation, and the assessment of the radioligand binding after various subsequent incubation times. Briefly, if the competitor dissociates at a comparable or faster rate than the radioligand, the radioligand binding curve follows a hyperbolic trend until equilibrium is reached. If the competitor dissociates more slowly than the radioligand, the kinetic association curve exceeds its equilibrium for a certain time, then decreases up to reach equilibrium. As a result, the association (k_{on}) and dissociation (k_{off}) rate constants of the competitor are derived, and then the parameter RT ($1/k_{off}$, expressed in minutes) is calculated from k_{off}.

RESULTS AND DISCUSSION

In Vitro Neurosteroidogenic Activity of Etifoxine. As a first step, the neurosteroidogenic ability of etifoxine was investigated in a well-validated neurosteroidogenic cell model (rat glioma C6 cells), using experimental conditions commonly employed in our previous studies.[15,16] The first metabolite of neurosteroidogenesis, pregnenolone (released from cells), was quantified following 2 h cell exposure with increasing ligand concentrations in serum-free conditions, and in the presence of further pregnenolone metabolism inhibitors. According to this method, TSPO ligands could be defined as "high steroidogenic ligands" showing a maximum efficacy (E_{max}) value of 250% or more (vs control, set up at 100%), or "low steroidogenic ligands" showing an E_{max} value of 140−150% (vs control, set up at 100%).[15] The classical selective TSPO ligands, PK11195 and Ro5-4864 (low steroidogenic ligands), were tested in parallel experiments for comparison purposes. As shown in Figure 1, etifoxine resulted more effective in stimulating pregnenolone production than PK11195 and Ro5-4864. In particular, etifoxine induced a dose-dependent pregnenolone production, reaching 235 ± 18% efficacy at the highest tested 100 μM concentration (vs control, set up at 100%). The observed etifoxine neurosteroidogenic efficacy was in agreement with previously published results, although obtained using different etifoxine incubation time and concentration (24 h, 10 μM etifoxine: approximately 220% efficacy. To assess whether the etifoxine neurosteroidogenic effect was affected by the potential interaction of the drug with the $GABA_A$ receptor, the presence of the receptor was investigated in C6 cells by the use of the selective $GABA_A$ receptor radioligand [^{3}H]flumazenil. The specific [^{3}H]flumazenil binding was not detectable, suggesting that $GABA_A$ receptor, at protein level, is not expressed in C6 cells, in agreement with literature data.[22]

Thermodynamic and Residence Time Parameters of Etifoxine at TSPO [^{3}H]PK11195 Binding Site. At first, the etifoxine thermodynamic equilibrium parameter K_i (inhibitory constant, index of binding affinity) was measured in rat kidney membranes, a TSPO-rich tissue source. The K_i value was 14.1 ± 2.8 μM, (Figure 2A) in agreement with previous results (etifoxine concentration that inhibits 50% of [^{3}H]PK11195 binding, IC_{50} = 27.3 μM and 18.3 μM in rat heart and forebrain, respectively).[6,8] Nanomolar K_i values at [^{3}H]PK11195 binding site for the classical TSPO ligands have been previously

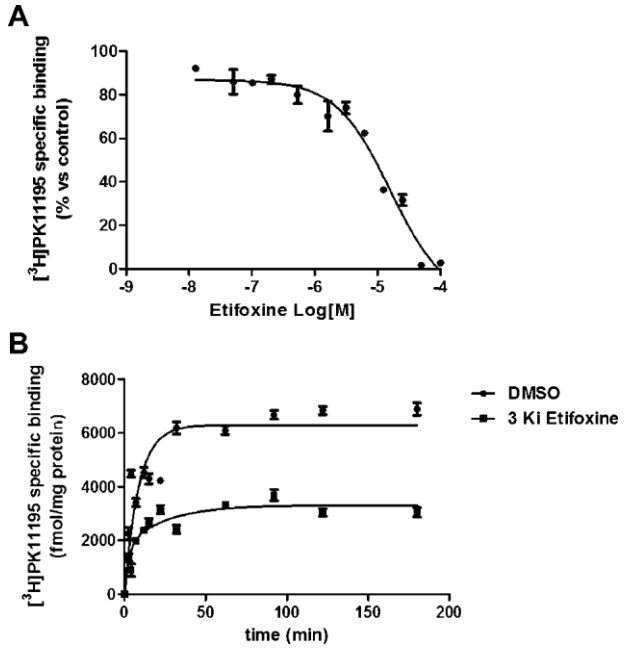

Figure 2. Etifoxine binding parameters at [^{3}H]PK11195 binding site in rat kidney membranes. (A) Displacement curve of [^{3}H]PK11195 specific binding to TSPO by etifoxine. The ordinate reports the [^{3}H]PK11195 specific binding obtained in the presence of etifoxine and calculated as percentage of control ([^{3}H]PK11195 specific binding obtained in the absence of etifoxine). The abscissa reports the etifoxine logarithmic concentration. The "log(inhibitor) vs response" analysis of the GraphPad computer program was used to fit the concentration–response curve and derive etifoxine IC_{50}. IC_{50} was converted to K_i value using the method of Cheng and Prusoff.[33] The data points represent the means ± SEM of three independent experiments, each performed in triplicate. The K_i value was 14.1 ± 2.8 μM. (B) Etifoxine RT at [^{3}H]PK11195 binding site by "competitive kinetics of association" assay. Representative curves obtained by incubation of rat kidney membranes with either radioligand alone or radioligand and etifoxine (tested at 3-fold K_i) for the indicated time points. The etifoxine RT mean ± SEM (15 ± 2 min) was calculated from three independent experiments.

determined (PK11195 and Ro5-4864, K_i of 3.4 and 20.0 nM, respectively).[15]

Then, etifoxine RT at [^{3}H]PK11195 binding site was estimated in rat kidney membranes, applying the "competition kinetic association" method, and using a 3-fold K_i concentration of etifoxine. As shown in Figure 2B, the "competition kinetic association" curve in the presence of etifoxine follows a hyperbolic trend until equilibrium is reached, in line with the trend of a ligand that dissociates from the binding site similarly or faster than the radioligand. The etifoxine kinetic parameters were k_{on}= 5.7 ± 0.7 x10^3 M^{-1} min^{-1}, k_{off} = 0.067 ± 0.009 min^{-1}, and the derived RT was 15 ± 2 min. Taking into consideration our recently published data, etifoxine showed a RT value at PK11195 binding site in line with RTs of "low neurosteroidogenic" ligands (approximately 150% neurosteroidogenesis increase vs control, set up at 100%; RT = 10−30 min), rather than with those of "high neurosteroidogenic" ligands (approximately 250% neurosteroidogenesis increase vs control, set up at 100%); RT = 100 min).[15,16] Due to the well-known etifoxine high neurosteroidogenic efficacy, this unexpected result prompted us to investigate etifoxine kinetic binding parameters at Ro5-4864 binding site. Several evidence supported that Ro5-4864 binding site is not identical to the PK11195 one.[23–25] Although the molecular determinants underlying interaction of PK11195 and Ro5-4864 with TSPO are not yet fully understood, heterogeneous sites for these two ligands, either partially overlapping or allosterically coupled, have been proposed.[23–25]

Thermodynamic and Residence Time Parameters of Etifoxine at TSPO [^{3}H]Ro5-4864 Binding Site. Thermodynamic and kinetic parameters of etifoxine at [^{3}H]Ro5-4864 binding site were investigated in rat kidney membranes. To this aim, the following experimental strategy was undertaken:

Etifoxine K_i Determination. To assess etifoxine affinity at [^{3}H]Ro5-4864 binding site, K_i was determined by displacement competition assay. The results demonstrated that etifoxine inhibited [^{3}H]Ro5-4864 binding in a dose-dependent manner, giving a K_i value of 9.0 ± 0.9 μM (Figure 3A).

Etifoxine Competitive or Noncompetitive Binding Evaluation. Saturation analysis of [^{3}H]Ro5-4864 binding was performed in the presence and in the absence of etifoxine (at K_i concentration) to assess if etifoxine competitively or noncompetitively binds to Ro5-4864 binding site. Scatchard analysis of the [^{3}H]Ro5-4864 binding saturation data revealed that etifoxine caused a decrease in receptor affinity (measured by the equilibrium dissociation constant K_d), but no effect on density of binding sites (B_{max}) (with etifoxine: K_d = 22.2 ± 1.7 nM, B_{max} = 3501 ± 174 fmol/mg protein; without etifoxine: K_d = 11.1 ± 1.1 nM, B_{max} = 3498 ± 114 fmol/mg protein. This result indicated an apparently competitive interaction of etifoxine at [^{3}H]Ro5-4864 binding site in rat kidney membranes (Figure 3B).

Etifoxine Kinetic Parameter Determination. Kinetic parameters were measured by the "competition kinetic association" method, in order to calculate etifoxine RT at [^{3}H]Ro5-4864 binding site. As shown in Figure 3C, [^{3}H]Ro5-4864 "competition kinetic association" curve exceeds its equilibrium for a certain time, then decreases to reach equilibrium, following a pattern typical for a competitor with slower dissociation rate than radioligand. The obtained kinetic parameters of etifoxine were k_{on} = 5.0 ± 0.4 × 10^3 M^{-1} min^{-1}, and k_{off}= 0.020 ± 0.002 min^{-1} (Figure 3C). The etifoxine RT was calculated to be 50 ± 5 min. The Ro5-4864 RT on TSPO resulted to be 16 min, as calculated from previously reported k_{off} values.[26,27]

Taken together, the present results provide useful information to shed light on the mechanism by which the approved anxiolytic drug etifoxine increases endogenous neurosteroid levels. Actually, it has been questioned that such an effect could derive from an interaction with the steroidogenic protein 18 kDa TSPO, as etifoxine binds to TSPO with low micromolar affinity;[6,8] and the classical TSPO ligand PK11195, which generally blocks the effects triggered by other TSPO ligands, does not abolish the etifoxine-induced neurosteroid production in frog hypothalamus.[14] On the other hand, in support of a TSPO specific contribution, etifoxine steroidogenic activity resembles that of some ligands interacting with TSPO,[8,9,12,15,16,28,29] and PK11195 only partly suppresses the etifoxine-induced effects on $GABA_A$ receptors in rat hypothalamic cultures.[6] However, the lack of a total abolition of these effects by PK11195 could be due to the etifoxine ability to modulate the $GABA_A$ receptor also by means of a direct interaction with this receptor.

Etifoxine binds with a micromolar affinity (in term of the thermodynamic parameter at equilibrium K_i) and non-competitively at PK11195 site, suggesting partially overlapping

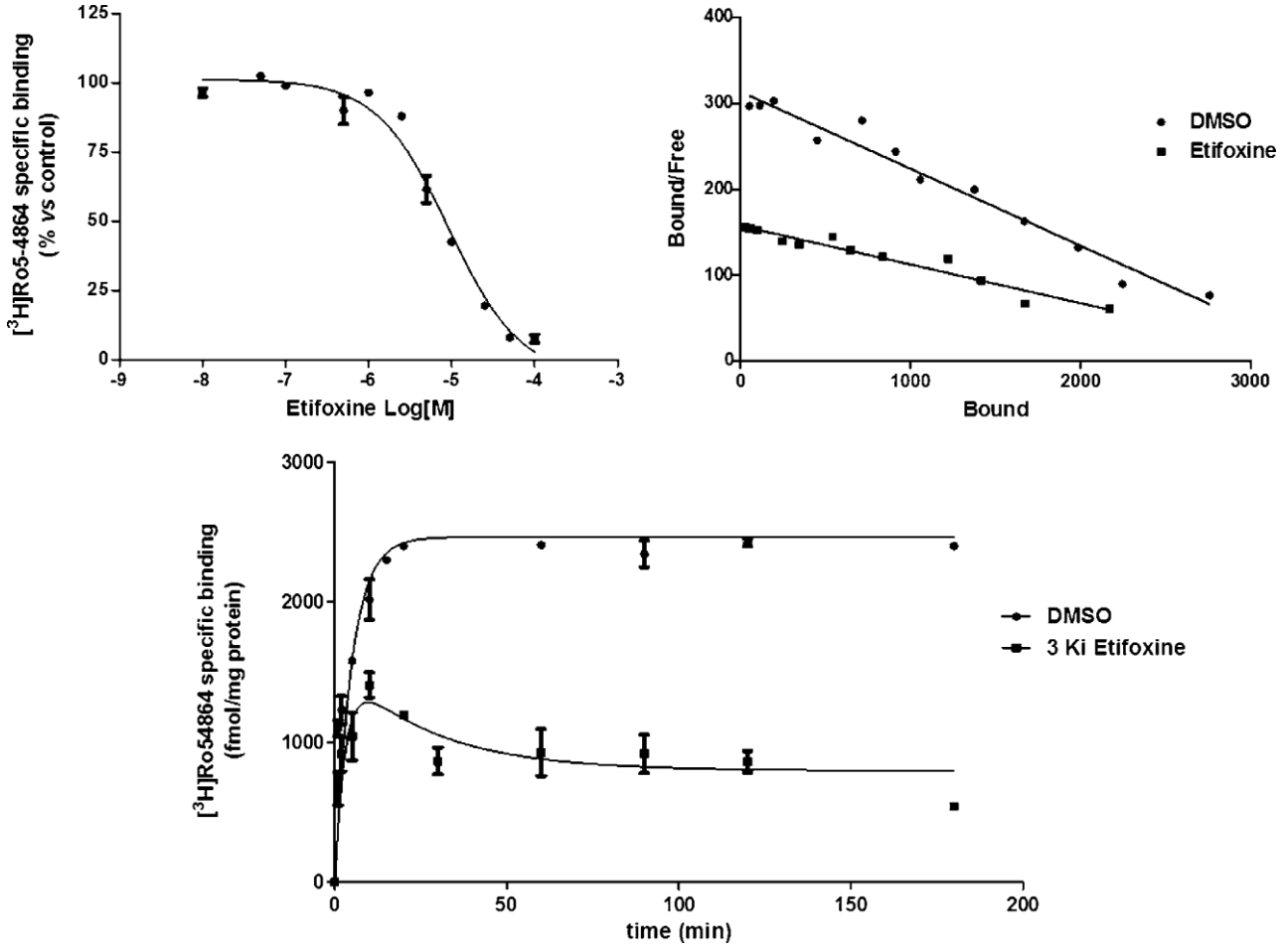

Figure 3. Etifoxine binding parameters at [^{3}H]Ro5-4864 binding site in rat kidney membranes. (A) Displacement curve of [^{3}H]Ro5-4864 specific binding to TSPO by etifoxine. The "log(inhibitor) vs response" analysis of the GraphPad computer program was used to fit the concentration–response curves and derive etifoxine IC_{50}. IC_{50} was converted to K_i value using the method of Cheng and Prusoff.[33] K_i value was 9.0 ± 0.9 μM. The data points represent the means ± SEM of three independent experiments, each performed in triplicate. (B) [^{3}H]Ro5-4864 binding saturation data were fitted to eq 2. Values shown are means from a single representative experiment performed in duplicate for each sample (with or without etifoxine). The K_d and B_{max} means ± SEM were determined from three independent experiments: with etifoxine, K_d = 22.2 ± 1.7 nM, B_{max} = 3501 ± 174 fmol/mg protein; without etifoxine: K_d = 11.1 ± 1.1 nM, B_{max} = 3498 ± 114 fmol/mg protein. (C) "Competition kinetic association" of etifoxine at [^{3}H]Ro5-4864 binding site. Representative curves obtained by incubation of membranes with either radioligand alone or radioligand and etifoxine (tested at 3-fold K_i) for the indicated time points. The etifoxine RT was 50 ± 5 min.

binding sites for the two ligands.[6,8] Indeed, in the present work, a micromolar binding affinity of etifoxine to this TSPO binding site was confirmed (approximately 4000-fold lower than PK11195). The etifoxine K_i at the site for the other classical TSPO ligand Ro5-4864 was investigated for the first time, highlighting a micromolar affinity (approximately 800-fold lower than Ro5-4864). In addition, the interaction of etifoxine with the Ro5-4864 site resulted competitive, supporting the hypothesis of an identical binding site for the two ligands. Despite the low affinity at both TSPO binding sites, etifoxine showed higher in vitro neurosteroidogenic efficacy than PK11195 and Ro5-4864. These data confirmed that the parameter K_i (quantified at PK11195, but also at Ro5-4864 site) is not the best in vitro measurement that accounts for the neurosteroidogenic effectiveness of etifoxine. This phenomenon is common among other TSPO ligands, thus indicating a poor relationship between binding affinity and neurosteroidogenic efficacy.[18,30] Our recent data have suggested that a long "Residence Time" (RT) of a ligand to TSPO is crucial to predict the ability to stimulate neurosteroidogenesis, irrespective of the binding affinity.[15,16] The results of the present study highlight RT at TSPO as the relevant in vitro parameter for neurosteroidogenic efficacy also for etifoxine. In particular, etifoxine showed a long RT at the Ro5-4864 site, approximately 3-fold longer than Ro5-4864. This trend is consistent with that showed by the candidate anxiolytic XBD173 and some PIGAs with promising anxiolytic activities in animal models, that exhibited a longer (approximately 3- or 4-fold longer) RT than PK11195 at the PK11195 site. Etifoxine showed a RT more in line with that of poorly neurosteroidogenic TSPO ligands at PK11195 site, strongly suggesting that etifoxine stimulates neurosteroidogenesis via the Ro5-4864 binding site. Our results suggest that despite the binding assay and the steroidogenic assay are conducted at two different temperatures for experimental requirements, the residence time parameter keeps the characteristics of a predictive parameter for the ligand efficacy in in vitro steroidogenesis.

In conclusion, the present results further corroborate the literature data suggesting TSPO as a molecular target of etifoxine. The long RT of etifoxine at Ro5-4864 binding site could be the mechanism by which it promotes neurosteroidogenesis, again supporting the relevance of RT as a predictive

measure of neurosteroidogenic efficacy for TSPO ligands. Actually, it might be proposed that an efficacious pharmacological stimulation of neurosteroidogenesis could be obtained by the use of a TSPO ligand that interacts with a long residence time at PK11195 (at least 100 min) or at Ro5-4864 (at least 50 min) binding site. This has important implications, as the pharmacological stimulation of neurosteroidogenesis via TSPO could represent a suitable strategy to obtain promising anxiolytic agents, devoid of the typical adverse effects of BDZs.

METHODS

Pregnenolone Measurement. Pregnenolone production was evaluated using rat glioma C6 cells, as previously described.[15,16] Briefly, C6 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 U/mL streptomycin and maintained in humidified atmosphere of 5% CO_2 and 95% air, at 37 °C. For each experiment, C6 cells were seeded in 96-well plate (10^4 cells/well) in a final volume of 100 μL of complete medium. Following 24 h, the complete medium was removed and cells were incubated for 2 h with a salt buffer (140 mM NaCl, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 10 mM glucose, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgSO_4$, pH 7.4) supplemented with 0.1% bovine serum albumin and inhibitors of further pregnenolone metabolism (25 μM trilostane and 10 μM SU10603) in the presence of increasing concentration (0.1 nM−100 μM) of etifoxine, Ro5-4864 or PK11195. The solvent (DMSO and ethanol) concentrations did not exceed 0.5% (v/v). At the end of the incubation time, the conditioned salt buffer was collected and the pregnenolone concentrations were measured by ELISA.

Radioligand Binding Assays. [^{3}H]Flumazenil binding assay was performed using rat C6 glioma membranes. For crude membrane preparation, C6 cells were harvested using phosphate buffer saline (PBS), pH 7.4, supplemented with EDTA 0.04% and centrifuged at 1000*g* for 10 min. The obtained pellet was suspended in ice-cold buffer (Tris−HCl 5 mM, pH 7.4) containing protease inhibitors (160 μg/mL benzamidine, 200 μg/mL bacitracin and 20 μg/mL trypsin inhibitor), homogenized with an Ultraturrax, and centrifuged at 48 000*g* for 15 min at 4 °C. Then, the resulting pellet was resuspended in Tris−HCl 50 mM, pH 7.4, and an additional centrifugation step followed (48 000*g*, 15 min, 4 °C). The resulting cell membrane pellet was suspended in Tris-citrate 50 mM, pH 7.4 and homogenized by using an Ultraturrax homogenizer. [^{3}H]PK11195 and [^{3}H]Ro5-4864 binding assays were performed using rat kidney membranes. Rat kidney membranes were prepared as previously reported,[31] aliquoted and stored at −20 °C until further use. For radioligand binding assays, an aliquot of membrane pellet was thawed, suspended in Tris-HCl 50 mM, pH 7.4 and homogenized by using an Ultraturrax homogenizer. The experimental procedures were performed following the guidelines of the European Community Council Directive 86-609 and approved by the Committee for animal experimentation of the University of Pisa.

For all radioligand binding assays, the protein content in membrane homogenates was determined by the Bradford method using the Bio-Rad Protein Assay Dye reagent.[32] For all radioligand binding assays, incubation times of each sample were terminated by filtration under vacuum using GF/C glass fiber filters. After two washing with 4 mL of ice-cold assay buffer, radioactivity was measured by liquid scintillation counter (TopCount; PerkinElmer Life and Analytical Sciences; 65% counting efficiency).

[^{3}H]Flumazenil Binding Assay. Membrane homogenates (20 μg of proteins) were incubated with 0.4 nM [^{3}H]flumazenil (specific activity 85.4 μCi/nmol) in 500 μL final volume of Tris-citrate 50 mM for 90 min at 0 °C. Nonspecific [^{3}H]flumazenil binding was determined in the presence of 50 μM diazepam.

[^{3}H]PK11195 Binding Assays. For displacement binding assay, an aliquot of membrane pellet was thawed, suspended in Tris−HCl 50 mM, pH 7.4 and homogenized by Ultraturrax. Membrane homogenates (15 μg of proteins) were incubated with increasing concentrations of etifoxine (10 nM to 100 μM) and 0.5 nM [^{3}H]PK11195 (Specific Activity 80.9 μCi/nmol) in 500 μL final volume of Tris-HCl 50 mM, pH 7.4 for 90 min at 0 °C. Nonspecific [^{3}H]PK11195 binding was determined in the presence of 1 μM PK11195. The solvent (ethanol) concentration was less than 1% in each sample. For "competition kinetic association" assay, the previously reported experimental conditions were used.[15] The assay was performed using concentration of etifoxine corresponding to 3-fold its K_i.

[^{3}H]Ro5-4862 Binding Assays. For displacement binding assay, membrane homogenates (60 μg of proteins) were incubated with increasing concentrations of etifoxine (10 nM to 100 μM) and 0.3 nM [^{3}H]Ro5-4864 (Specific Activity 49.4 μCi/nmol) in 500 μL, the final volume of Tris-HCl 50 mM, pH 7.4 for 90 min at 4 °C. Nonspecific [^{3}H]Ro5-4864 binding was determined in the presence of 50 μM diazepam. For saturation assay, membrane homogenates (60 μg of proteins) were incubated with increasing [^{3}H]Ro5-4864 concentrations (0.05−35 nM; Specific Activity, 49.4 μCi/nmol) in the presence and in the absence of 10 μM etifoxine in 500 μL final volume of Tris−HCl 50 mM, pH 7.4 for 90 min at 4 °C. Nonspecific [^{3}H]Ro5-4864 binding was obtained in the presence of 50 μM diazepam. For "competition kinetic association" assay, [^{3}H]Ro5-4864 (approximately 35 nM; specific activity 24.7 μCi/nmol) and etifoxine (3-fold its K_i) were simultaneously added to membrane homogenates (60 μg of proteins) in 500 μL final volume of Tris−HCl 50 mM, pH 7.4. The incubation was conducted at 4 °C and terminated at multiple time points.

Data Analysis. The experiments were analyzed by linear or non linear regression using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA). The concentration of etifoxine that inhibited by 50% [^{3}H]Ro5-4864 or [^{3}H]PK11195 binding to kidney membranes (IC_{50}) was calculated by fitting the data to the "log(inhibitor) *vs*. response equation" (GraphPad Software Inc., San Diego, CA):

$$Y = \text{bottom} + \frac{\text{top} - \text{bottom}}{1 + 10^{(x - \log IC_{50})}} \quad (1)$$

where "bottom" is the maximally inhibited response and "top" is the maximal response. IC_{50}'s were converted to K_i value using the method of Cheng and Prusoff.[33]

Scatchard plot analysis[34] was performed to calculate maximum binding sites (B_{max}) and equilibrium constant (K_d) values of [^{3}H]Ro5-4864 at TSPO. Data obtained by the saturation radioligand binding experiments were fitted to the equation:

$$\frac{B}{F} = -\frac{B}{K_d} + \frac{B_{max}}{K_d} \quad (2)$$

which fits the equation of a line ($y = mx + b$; B/F vs B) where B_{max}/K_d is the y-intercept, B_{max} is the x-intercept, and $-1/K_d$ is the slope. B is the radioligand bound to the protein, and F is the radioligand in the free form.

Etifoxine k_{on} and k_{off} at [^{3}H]Ro5-4864 or [^{3}H]PK11195 binding sites were calculated applying the competition association model using the "kinetics of competitive binding" assay, as previously reported.[15] In such a model, to derive k_3 and k_4 values of etifoxine (k_{on} and k_{off} values, respectively), the previously reported k_1 and k_2 of [^{3}H]PK11195 and [^{3}H]Ro5-4863[27] binding were used.

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Author Contributions

B.C. conceived the idea, analyzed the results, and wrote the manuscript. C.C. conducted the experiments, contributed to write the manuscript, and analyzed the results. E.D.P. analyzed the results and revised the manuscript. S.T. revised the manuscript. F.D.S. revised the manuscript. C.M. conceived the idea of the manuscript and revised the manuscript.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

TSPO, translocator protein; RT, residence time; etifoxine, 2-ethylamino-6-chloro-4-methyl-4-phenyl-4H-3,1-benzoxazine hydrochloride; Ro5-486, 4′-chlorodiazepam; PIGAs, phenylindolylglyoxylamides; k_{on}, association rate constant; k_{off}, dissociation rate constant

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