Residence time (RT), a new parameter to predict neurosteroidogenic efficacy of Translocator Protein (TSPO) ligands: *N,N*-dialkyl-2-arylindol-3-ylglyoxylamides, a case study.

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Abstract

Targeting neuroactive steroid biosynthetic pathway by specific 18 kDa Translocator Protein (TSPO) ligands may represent a therapeutic approach in a variety of neurodegenerative and neuropsychiatric diseases. However, the lack of correlation between the binding affinity and the *in vitro* steroidogenic efficacy has limited the identification of lead compounds by a traditional affinity-based drug discovery strategy. Our recent researches indicate that the key factor for robust steroidogenic TSPO ligand efficacy is not the binding affinity *per se*, but rather the time the compound spends into the target, namely its Residence Time (RT). The assessment of this kinetic parameter during the *in vitro* characterization of compounds appears mandatory in order to obtain structure-efficacy relationships suitable for the future development of novel molecules with promising pharmacological properties.

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Neuroactive steroids are endogenous neuromodulators that, by binding to membrane receptors and tuning gene expression *via* intracellular receptors, regulate many physiological functions. They can be synthesized in the brain *de novo*, so that they were termed neurosteroids, or reach the central nervous system (CNS) from peripheral steroidogenic organs, such as adrenals and gonads, and are locally metabolized. Neurosteroids levels are altered in several psychiatric and neurodegenerative diseases as a consequence of an impairment of neurosteroidogenesis; both preclinical and clinical studies emphasize a therapeutic potential of neuroactive steroids for these diseases, whereby symptomatology ameliorates upon restoration of neuroactive steroid concentrations.[1-3]

Actually, neuroactive steroids exert potent anxiolytic, antidepressant, anticonvulsant, sedative, analgesic and amnesic effects, mainly acting as positive allosteric modulators at specific sites on the α-subunit of the γ-amino-butyric acid type A receptor ($GABA_AR$).[4] In addition, neuroactive steroids exert neuroprotective, neurotrophic, anti-inflammatory and antiapoptotic activities in several animal models of traumatic brain and spinal cord injury, cerebral ischemia, peripheral neuropathy, and neurodegenerative diseases (i.e. Alzheimer's and Parkinson's diseases, multiple sclerosis, etc...).[2]

However, direct administration of neuroactive steroids has several challenges, including short half-life, low bioavailability, poor aqueous solubility, development of tolerance, undesired effects such as sedation and memory impairment that limit their therapeutic use. Consequently, modulation of neurosteroidogenesis to restore the altered endogenous neuroactive steroid tone may represent a better therapeutic approach.[1-3]

Neuroactive steroid biosynthetic pathway may be targeted at different levels in order to promote neurosteroidogenesis,[3] including the Translocator Protein 18 kDa (TSPO), an outer mitochondrial membrane protein expressed at high levels in peripheral and CNS steroid-producing cells.[5]

TSPO plays a key role in the rate-limiting step of neuroactive steroid synthesis, consisting of cholesterol translocation into mitochondrion in order to supply it to the cytochrome P450 enzyme CYP11A1 for the conversion into pregnenolone, the precursor of all neurosteroids.[6]

Numerous TSPO ligands resulted able to potently and dose-dependently stimulate steroid biosynthesis in steroidogenic cells, and they have been proposed as innovative therapeutic tools in several pathological conditions, due to their neuroprotective, anxiolytic, anti-inflammatory, and regenerating properties in different *in vitro* and *in vivo* models.[7-10] To date, phase II clinical trials have been concluded for the treatment of diabetic peripheral neuropathy (ClinicalTrials.gov identifier: NCT00502515), and generalized anxiety disorder (NCT00108836).[11,12]

Since identification of TSPO by means of the benzodiazepines diazepam and Ro5-4864 (**1**) (Figure 1),[13] structurally different classes of highly potent and selective TSPO ligands have been reported,[14] including the isoquinolinecarboxamides, of which the 1-(2-chlorophenyl)-*N*-methyl-*N*-

(1-methylpropyl)-1-isoquinolinecarboxamide (PK11195, **2**, Figure 1) is widely considered as a prototypical TSPO ligand,[15] imidazopyridines (alpidem),[16] indoleacetamides (FGIN-1-27),[17] aryloxyanilides,[18] 4-phenylquinazolines,[19,20] and purineacetamides (XBD173, also called AC-5216 **13**, *vide infra*).[11,12]

In this context, we disclosed a class of potent and selective TSPO ligands, the *N*,*N*-dialkyl-2-phenylindol-3-ylglyoxylamides (PIGAs, **3-12**, Figure 1), the majority of which showed K_i values in the nanomolar/subnanomolar range; moreover, a number of compounds were able to stimulate effectively steroid biosynthesis.[21-23]

In two recent studies, a number of our PIGA ligands promoted the well-being of human astrocytes and prevented oxidative damage and inflammatory response in an *in vitro* neuroinflammatory model, suggesting these compounds could represent potential new therapeutic tools for the treatment of inflammatory-based neuropathologies and/or for CNS diseases characterized by astrocyte loss. Interestingly, in both cases the observed effects were completely counteracted by the co-treatment with DL-aminoglutethimide, an inhibitor of P450scc, supporting the hypothesis that the PIGA-mediated protective mechanisms were mainly related to steroid production.[25,26]

Furthermore, some PIGA compounds have been evaluated *in vivo* for their anxiolytic properties by means of the elevated plus-maze (EPM) tests in rats. Two compounds significantly affected rats' performance, leading to an increase in both entries and time spent in the open arms, with no effect on rats' spontaneous exploratory activity, evidencing promising anxiolytic/non sedative properties. Investigations on the mechanism of action by which PIGAs exert their anxiolytic activity indicate that it involves the stimulation of endogenous neurosteroid production, which in turn determines a positive modulation of $GABA_AR$ activity.[22,24]

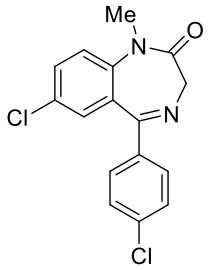
However, one of the most recurrently issue concerning TSPO ligands consists in the lack of correlation between the binding affinity and the *in vitro* efficacy, including steroidogenic efficacy. This represents a very crucial point, because this phenomenon has limited not only the identification of lead compounds by a traditional affinity-based drug discovery strategy, but also questioned the specificity of the observed effects.[27]

This is one of the most recurrent issue concerning TSPO ligands[27] and it's evident also within the PIGA class, with derivatives that, despite a very similar binding affinity, showed a great difference in steroidogenic efficacy, measured as the ability to stimulate *in vitro* pregnenolone formation. Analogously, compounds with a comparable efficacy show a discrepancy in K_i values.[21-23]

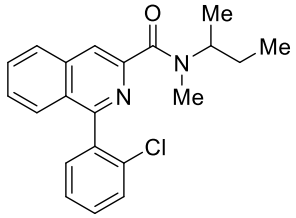
Recent studies have shown that the affinity of a ligand for its target could not directly define its biological effectiveness that may instead be related to the period that a drug resides on its target after

binding, namely its 'Residence Time' (RT).[28] In principle, the lifetime of the binary drug-target complex is determined by two rate constants, the association rate constant (K_{on}), and the dissociation rate constant (K_{off}), but the slow drug-target dissociation seemed to be the main critical molecular determinant for pharmacological activity. The relevance of RT, which is defined as the reciprocal of K_{off}, as a key factor for successful lead optimization processes has been documented for other ligand-target systems, including antagonists of adenosine A_{2A} and muscarinic M_3 receptors, several kinase inhibitors, HIV protease inhibitors, and so on.[28]

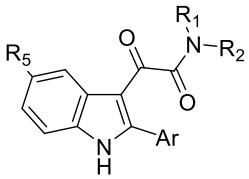
Thus, very recently we set up a kinetic radioligand binding assay for TSPO ligand RT determination.[29] A number of our previous reported TSPO ligands, belonging from the 2-phenylindolylglyoxylamide class (PIGAs), was selected based on their different abilities to stimulate *in vitro* steroidogenesis, compounds **3-12** Table 1.[21-23] Among such selected TSPO compounds, ligands possessing anxiolytic effects or *in vitro* pleiotropic and anti-inflammatory properties, as well as classical TSPO ligands **1** and **2** were included.



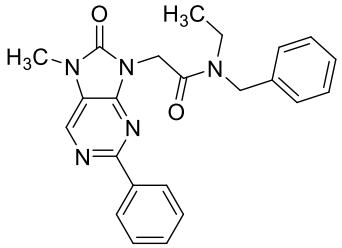
Ro5-4864 (1)



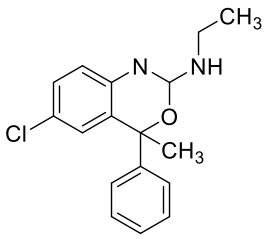
PK11195 (2)



PIGAs (3-12)



XBD173 (13)



Etifoxine (14)

Compd.	**R_5**	**Ar**	**R_1**	**R_2**
3 (PIGA719)[a]	H	C_6H_5	$(CH_2)_2CH_3$	$(CH_2)_2CH_3$
4 (PIGA720)[a]	H	C_6H_5	$(CH_2)_5CH_3$	$(CH_2)_5CH_3$
5 (PIGA745)[a]	Cl	C_6H_5	$(CH_2)_5CH_3$	$(CH_2)_5CH_3$
6 (PIGA823)[b,c]	Cl	C_6H_4-4-Cl	CH_2CH_3	$CH_2C_6H_5$
7 (PIGA835)[a]	Cl	C_6H_4-4-Cl	$(CH_2)_3CH_3$	$(CH_2)_3CH_3$
8 (PIGA839)[a,d]	H	C_6H_4-4-CH_3	$(CH_2)_2CH_3$	$(CH_2)_2CH_3$
9 (PIGA925)[b]	NO_2	C_6H_5	$(CH_2)_5CH_3$	$(CH_2)_5CH_3$

10 (PIGA1128)[e]	H	naphth-2-yl-	$(CH_2)_2CH_3$	$(CH_2)_2CH_3$
11 (PIGA1138)[e,f]	H	naphth-2-yl-	CH_3	$(CH_2)_4CH_3$
12 (PIGA1214)[e]	H	C_6H_4-4-COOH	$(CH_2)_5CH_3$	$(CH_2)_5CH_3$

[a] ref [21]; [b] ref [22]; [c] ref [25]; [d] ref [24]; [e] ref [23]; [f] ref [26]

Figure 1. Structures of Ro5-4864 (**1**), PK11195 (**2**), PIGAs (**3-12**), XBD173 (**13**), and Etifoxine (**14**).

To this aim, kinetic experiments were performed to measure K_{on} and K_{off} rate constants for each compound at [^{3}H]-**2** binding site, and their RT was calculated.[29] As it should be noted from the data reported in Table 1, the majority of the tested compounds resulted rapid dissociating competitors of **2** binding site (**3**, **4**, **5**, **7**, **9**, **12** and the reference **1**). Conversely, compounds **6**, **8**, **10**, and **11** were slow dissociating competitors.

Table 1. Experimental thermodynamic/kinetic data and steroidogenic parameter for Ro5-4864 (**1**), PK11195 (**2**), PIGAs (**4-13**), and XBD173 (**14**).

TSPO ligand	**Equilibrium K_i (nM)**	**RT (min)**	**Emax** (at 100 µM) (vehicle set to 100%)
1[a]	20.0 ± 2.0	32 ± 3	150 ± 4
2[a]	3.30 ± 0.3	34 ± 3	153 ± 4
3[a,b]	12.2 ± 1.0	11 ± 2	146 ± 2
4[a,b]	1.40 ± 0.2	26 ± 2	144 ± 4
5[a,b]	13.1 ± 1.1	17 ± 1	140 ± 5
6[a,c]	3.30 ± 0.3	127 ± 4	272 ± 11
7[a,b]	0.91 ± 0.1	17 ± 1	149 ± 4
8[a,b]	5.50 ± 0.4	109 ± 4	254 ± 5
9[a,c]	12.2 ± 3.1	15 ± 2	166 ± 5
10[a,d]	0.31 ± 0.02	55 ± 2	179 ± 7
11[a,d]	0.34 ± 0.03	141 ± 4	275 ± 5
12[a,d]	343.01 ± 15.94	39 ± 2	141 ± 4
13[e]	2.41	127	245 ± 17

data from ref.: [a], [29]; [b], [21]; [c], [22]; [d], [23]; [e], [31]

Notably, correlation analysis of the obtained results showed a highly significant positive correlation between the kinetic parameter RT and the compounds' efficacy (E_{max}) to stimulate *in vitro* steroidogenesis (Figure 2A).[29] In addition, highly significant correlation resulted also between the logarithm of RT and the area under the dose-response curve (AUC), a value that combines potency and efficacy of a drug into a single parameter (Figure 2B). On the contrary, no correlation was observed between these same parameters and the logarithm of K_i values (Figure 2C).[29]

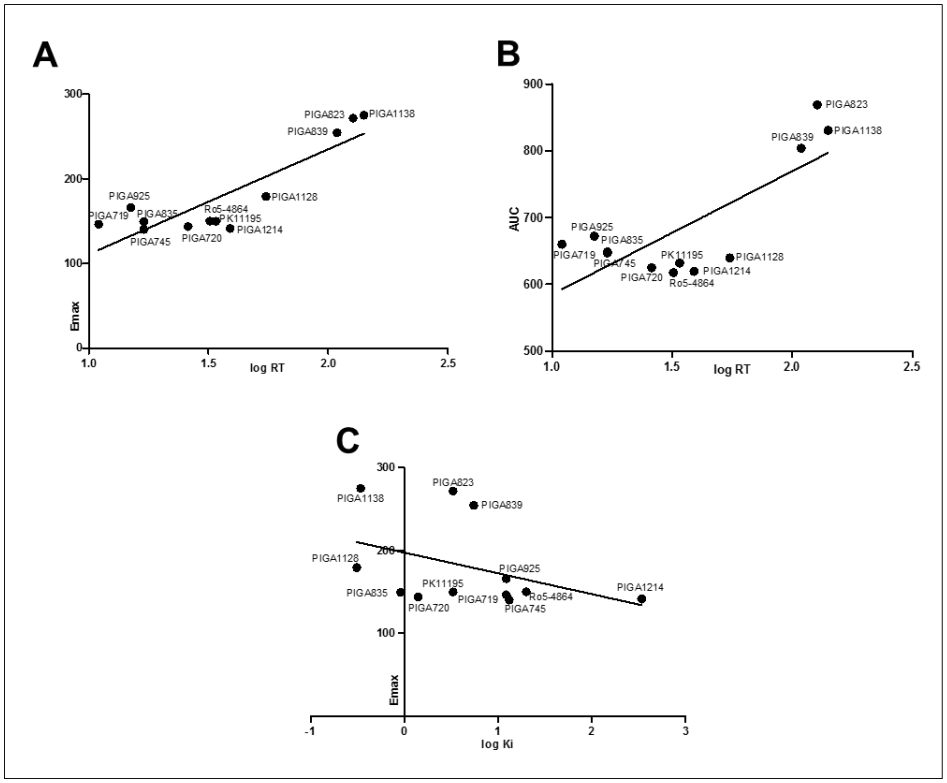


Figure 2. Correlation analyses between kinetic/thermodynamic and steroidogenic parameters. TSPO compound names were included next to their respective data points (for PIGA compounds the ID numbers were shown). (A) Scatter plot of the E_{max} values against kinetic parameters (log RT) of test TSPO ligands; (B) Scatter plot of the AUC values against kinetic parameters (log RT) of test TSPO ligands; (C) Scatter plot of the E_{max} values against thermodynamic parameters (log K_i) of test TSPO ligands. Adapted from ref [29].

From a therapeutic perspective, the "high neurosteroidogenic" **6** and **8** derivatives, characterized by long RT and high E_{max} (**6**: RT = 127 min, E_{max} = 272%; **8**: RT = 109 min; E_{max} = 254%), elicited a significant anxiolytic activity in the EPM paradigm in rat.[22,24] Consistently, **1** and **2** , which have been documented without anxiolytic activity in EPM test,[30] showed short RT and low Emax (**1**: RT= 32 min; E_{max} = 150%; **2**: RT= 34 min, E_{max} = 153%).[29]

Our hypothesis assessing that RT could be a metric of promising anxiolytic activities of a TSPO ligand found further support by very recent works, in which we retrospectively evaluate the RT and the relationship with steroidogenic activity of known anxiolytic TSPO ligands **13**[31] and Etifoxine (**14**)[32] (Figure 1, Table 1). This latter is a clinically approved drug for the treatment of anxiety-related disorders (Stresam, Biocodex, Gentilly, France).

Also for such compounds, a discrepancy between the affinity at [^{3}H]-**2** binding site and ability to enhance neurosteroid synthesis has been recently documented[33] and confirmed by us.[31,32] Specifically, although **13** and **14** are both highly potent to stimulate neurosteroidogenesis, **14** shows a very lower binding affinity to [^{3}H]-**2** site than **13**, questioning the specific contribution of TSPO in mediating **14** neurosteroidogenic efficacy.

When **13** was evaluated by kinetic assays using [^{3}H]-**2**, an approximately 4-fold longer RT (127 min) than [^{3}H]-**2** was derived (Table 1); consistently, **13** stimulated efficaciously neurosteroidogenesis, with E_{max} 245 ± 17% (Table 1), value comparable to that of the "high steroidogenic" PIGAs exhibiting anxiolytic effects in rats.[31]

In similar experiments, **14** induced a dose-dependent pregnenolone production (E_{max} 235 ± 18 %), combined with a surprising short RT at PK11195 (**2**) binding site (RT 15 ± 2 min), a value in line with RTs of "low neurosteroidogenic" ligands. This unexpected result prompted us to consider the existence on TSPO of two heterogeneous sites for reference ligands **1** and **2**, either partially overlapping or allosterically coupled.[34] At [^{3}H]-**1** binding site, **14** competitively bound with a low affinity (approximately 800-fold lower than **1**) and a long RT (RT 50 ± 5 min, approximately 3-fold longer than **1**).[32]

Based on these results, 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 benzodiazepines.

In conclusion, the lack of correlation between the compound binding affinity and the steroidogenic efficacy evidences the limitation of an affinity-based structure-activity relationships (SAR) strategy for the identification of effective TSPO ligands. Conversely, the time spent by a drug into its target (RT) represent a critical predictor for *in vitro* and, most importantly, *in vivo* efficacy. These findings represent a significant advancement in the TSPO medicinal chemistry field, highlighting that structure-efficacy relationships studies based on kinetic parameters are a more suitable approach for the development of novel compounds with promising pharmacological properties.

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Keywords: Translocator Protein, Residence Time, Neurosteroidogenesis, Structure-activity relationships.

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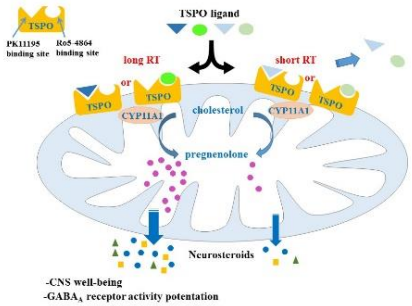
Graphical Table of Contents



Neurosteroidogenic efficacy of Translocator Protein (TSPO) ligands can be predicted by evaluating the time that the ligand spent into the target, namely the **Residence Time**, rather than the binding affinity, aiding the development of novel compounds with promising pharmacological properties and therapeutic potential.