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Mendeleev Commun., 2024, **34**, 682–684

Mendeleev Communications

Tripeptide Phe-Trp-Leu-NH_2 as a putative endogenous ligand of TSPO: molecular modeling, synthesis and pharmacological activity

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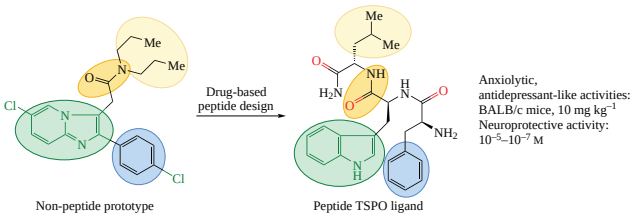
DOI: 10.1016/j.mencom.2024.09.018

The putative endogenous tripeptide ligand of the translocator protein (TSPO) L-phenylalanyl-L-tryptophanyl-L-leucine amide was obtained using the drug-based peptide design strategy and molecular modeling. This tripeptide demonstrated anxiolytic activity in the elevated plus-maze test and antidepressant-like activity in the forced swimming test in BALB/c mice at the doses of 10 mg kg^{-1} (*i.p.*) and also showed neuroprotective activity in the concentration range of 10^{-5}–10^{-7} M *in vitro* under conditions of oxidative stress using HT-22 neuronal cell line.



Keywords: tripeptide, TSPO ligand, anxiolytic activity, antidepressant-like activity, neuroprotective activity.

Anxiety disorders of different nature affect 6–10% of the world's population, making it necessary to design drugs that combine anxiolytic, antidepressant and neuroprotective actions. One of the promising targets for the development of well-tolerated neuropsychotropic drugs is the 18 kDa translocator protein (TSPO), which mediates mitochondrial cholesterol transfer and associated synthesis of neurosteroids.[1,2] Endogenous neurosteroids participate in both the excitatory (glutamate)[3] and the inhibitory (γ-aminobutyric acid)[4] transmission modulating different brain-related emotions and can, in particular, cause anxiolytic, antidepressant-like[5,6] and neuroprotective[7,8] effects. Currently, a number of endogenous and synthetic TSPO ligands are known which differ in their functional activity.[9–12] Thus, endogenous neuropeptide TTN ($DBI_{17–50}$) promotes neurosteroidogenesis and causes anxiety-related behavior,[13] synthetic compound PK11195[14] (Figure 1) acts as high affinity TSPO antagonist,[15] while structurally similar Alpidem[12] appears to stimulate neurosteroid production,[16] but, unlike TTN, has a pronounced anxiolytic effect.[17] This difference in properties as well as detailed mechanisms of action of TSPO interacting compounds remain unclear, due in part to the lack of knowledge about the endogenous ligand with Alpidem-like activity. It is logical to assume that some endogenous peptide can be a candidate for this role, but searching for it is a non-trivial task.

In our previous works, using a 'drug-based peptide design' approach[18] for Alpidem we obtained a series of substituted dipeptides[19,20] and revealed that *N*-phenylpropionyl-L-tryptophanyl-L-leucinamide [$Ph(CH_2)_2C(O)$-L-Trp-L-Leu-NH_2, code GD-102] possessed extremely high TSPO-mediated anxiolytic activity.[20] The data obtained allow us to suggest that the putative TSPO endogenous ligand may have a tripeptide structure similar to GD-102, namely L-Phe-L-Trp-L-Leu-NH_2 (code FWL-NH_2, see Figure 1). Phenylalanine, tryptophan and leucine moieties of such a peptide can imitate two aromatic nuclei and branch aliphatic chain of Alpidem. To test this assumption, molecular modeling, synthesis and evaluation of pharmacological activity spectrum of FWL-NH_2 were undertaken in this work.

The binding site of Alpidem is unknown, but considering the structural similarity of Alpidem and PK11195 it is logical to assume that they should occupy close regions in the protein. So, molecular docking and molecular dynamics for FWL-NH_2 were carried out using the model of the PK11195 binding site and adjacent regions in TPSO (PDB ID: 2MGY).[21] Modeling was

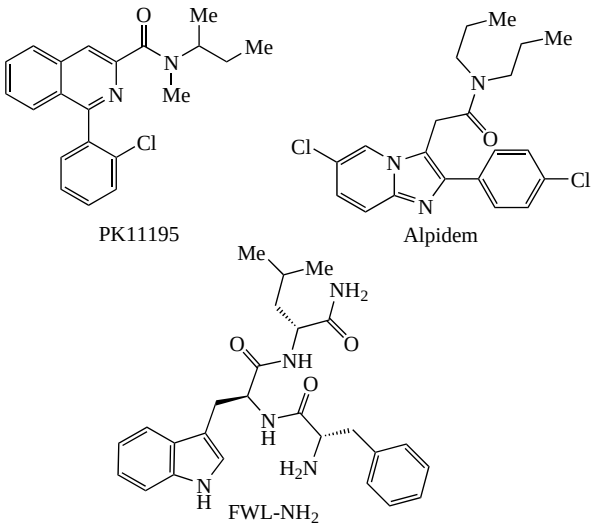
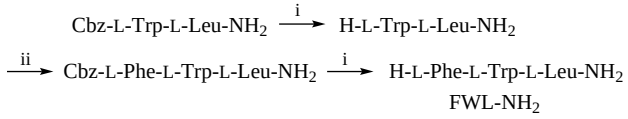


Figure 1 The structures of earlier described TPSO ligands PK11195,[14] Alpidem[17] and tripeptide L-Phe-L-Trp-L-Leu-NH_2 (FWL-NH_2) studied in the present work.

performed using both Glide software version 2022-4 build 134 from Schrödinger[22] and AutoDock Vina 1.1.2[23] software according to procedures described in refs. 20 and 24, respectively, and molecular dynamics was fulfilled using CHARMM36/CGenFF 4.4 force field[25,26] in GROMACS 2021.2 software[27] (for details, see Online Supplementary Materials). According to docking data, the location of tripeptide FWL-NH_2 is rather close to that of PK11195 due to the interactions of aromatic moieties of tripeptide with amino acid residues Trp 143 and Trp 107 and location of the Leu side chain of FWL-NH_2 near hydrophobic residues in the protein [Figure 2(*a*)]. Molecular dynamics simulations [see the plot of the root mean square deviations from the initial positions for the protein and ligand non-hydrogen atoms at Figure 2(*b*)] show that dynamic equilibrium is achieved after 20 ns and confirm (together with visual analysis) that the system retains stability over the following course of the production simulation (total 100 ns). As seen from Figure 2(*a*), the position of the tryptophane–leucine backbone of FWL-NH_2 remained virtually unchanged compared to the one obtained by molecular docking, while the phenylalanine residue shifted away from the Trp 107, but acquired the ability to π–π stacking with Phe 99 in the binding site (see Online Supplementary Materials, Figure S2). The different arrangement of the benzene rings corresponds to the assumption of different functional activities of PK11195 and FWL-NH_2. Besides, demonstrated constancy of the arrangement of the L-tryptophane-L-leucine backbone is in accordance with previously obtained data on the L-Trp-L-Leu key role for the anxiolytic activity of Alpidem analogue GD-102 (structurally similar to FWL-NH_2, see above).[20]

The tripeptide FWL-NH_2 was synthesized as shown in Scheme 1 by classical peptide synthesis in solution using the Anderson's method of carboxy group activation with succinimide.[29] The substituted dipeptide Cbz-L-Trp-L-Leu-NH_2 was prepared as described previously.[20] The total yield was 18% (for characterization, see Online Supplementary Materials).

The pharmacological analysis of the synthesized compound included primarily the study of anxiolytic activity using the elevated plus-maze test (EPM)[30] based on rodents' innate preference for enclosed spaces over open spaces. The compound



Scheme 1 *Reagents and conditions*: i, H_2, Pd/C, MeOH, room temperature, 3 h; ii, Cbz-L-Phe-OSu, DMF, room temperature, 12 h.

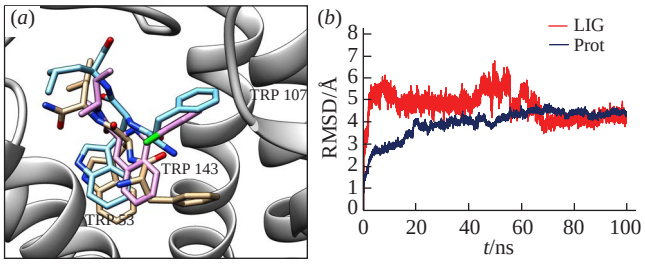


Figure 2 (*a*) Location of tripeptide FWL-NH_2 in the PK11195 binding site in TPSO (PDB ID: 2MGY) as predicted by molecular docking using Autodock Vina 1.1.2 (represented by a light blue-colored stick model) and the binding mode refined using molecular dynamics simulation (shown by beige stick model). The position of PK11195 is matched as a pink-colored stick model for comparison (visualization in UCSF Chimera[28]). The lipid molecules and hydrogen atoms are omitted. (*b*) Mass-weighted root mean square deviation (RMSD) of the non-hydrogen atoms of the FWL-NH_2/protein/membrane/water/ions system (shown in blue) and ligand FWL-NH_2 (shown in red) during molecular dynamics simulation of the complex with TSPO.

FWL-NH_2 at the dose of 10 mg kg^{-1} with single *i.p.* administration statistically significantly increased the percentage of open arms entries approximately threefold compared to the control group and caused a tendency to increase the percentage of time spent on the open arms (see Online Supplementary Materials, Table S1). Since these two parameters are considered as the main indicators of the anxiolytic effect,[31] it can be concluded that the compound FWL-NH_2 at the dose of 10 mg kg^{-1} (*i.p.*) exhibited anxiolytic activity in the EPM test in mice. The lower efficiency of the tripeptide FWL-NH_2 compared to the *N*-acyl-substituted dipeptide GD-102 (see Table S1) can be explained by the tripeptide's susceptibility to *N*-terminal cleavage by aminopeptidases due to the presence of an unsubstituted amino group.

Next, compound FWL-NH_2 was tested for antidepressant-like activity in a forced swim test in mice[32] based on the observation that a mouse placed in a situation of unavoidable swimming eventually stops making attempts to get out and takes a characteristic posture of immobility interpreted as despair. At the studied doses of 10 and 30 mg kg^{-1} (*i.p.*) FWL-NH_2 statistically significantly reduced the time of immobility of mice compared to the control group by 26% ($p = 0.019$) and 32% ($p = 0.002$), respectively (see Online Supplementary Materials, Table S2). The effect of FWL-NH_2 was about 60% of the effect of the classical antidepressant Amitriptyline at the dose of 10 mg kg^{-1} (*i.p.*).

Thus, compound FWL-NH_2 in the doses 10 and 30 mg kg^{-1} with single *i.p.* administration exhibits antidepressant-like activity in the forced swimming test in mice. Interestingly, FWL-NH_2 demonstrated anxiolytic and antidepressant-like activity in mouse models at the same doses.

The neuroprotective activity of FWL-NH_2 was studied in the model of oxidative stress using mouse hippocampal HT-22 cells.[33] Hydrogen peroxide statistically significantly reduced cell viability by approximately 30% compared to the control (Table 1). Tripeptide FWL-NH_2, when administered 24 h prior to hydrogen peroxide exposure, significantly improved cell survival in the concentration range of 10^{-5}–10^{-7} M. The activity had a dome-shaped dose dependence that is typical for peptides. We suppose that the presence of the neuroprotective effect of FWL-NH_2 for 24 h before injury may be associated with its effect on the activation of *de novo* synthesis of neurosteroids.

In general, we demonstrated that the obtained tripeptide L-Phe-L-Trp-L-Leu-NH_2 exhibited pronounced anxiolytic, antidepressant and neuroprotective properties, most likely through action on TSPO (taken into account its structural similarity to TSPO ligand GD-102). This allows one to expect that the specified tripeptide FWL-NH_2 may be putative endogenous TSPO ligand and makes its further detection in the course of *in*/*ex vivo* experiments interesting. Two peptides in the EROP-Moscow database,[34] namely Gastrin/cholecystokinin like peptide D1 and Natalisin 2 contain the required amino acid sequence (FWL) and may be endogenous precursors for the L-Phe-L-Trp-L-Leu-NH_2 tripeptide.

Table 1 Neuroprotective effect of FWL-NH_2 in the model of oxidative stress in HT-22 cells (MTT test).

Experimental group	*C*/mol dm^{-3}	Optical density	Activity (%)
Control	0	0.110 ± 0.004	100 ± 4
H_2O_2	1.5×10^{-3}	0.074 ± 0.006	67 ± 5[a]
FWL-NH_2	10^{-5}	0.085 ± 0.003	77 ± 3[b]
	10^{-6}	0.086 ± 0.004	78 ± 4[b]
	10^{-7}	0.086 ± 0.006	78 ± 5[b]
	10^{-8}	0.081 ± 0.005	73 ± 4

[a] $p \leqslant 0.05$ compared to passive control. [b] $p \leqslant 0.05$ compared to active control (H_2O_2, Kruskal–Wallis test followed by Dunn's test).

This study was conducted under the government contract of the Ministry of Science and Higher Education of the Russian Federation (grant no. FGFG-2022-0005).

Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.mencom.2024.09.018.

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Received: 3rd May 2024; Com. 24/7489