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

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1. Molecular modeling

Molecular docking was performed using the crystal structure of TSPO with ligand PK11195 (PDB ID: 2MGY). Ligand structure and protein model were prepared both as described in[S1] using the XP protocol in Glide software version 2022-4 build 134 from Schrödinger [ligand conformations were calculated in the LigPrep module in Maestro 13.5, grid coordinates x 5.65; y 4.66; z 5.91; 20Å) were centered to ligand] and as described in[S2] using the AutoDock Vina 1.1.2 software[S3] (grid box 11.25 Å×11.25 Å×11.25 Å, grid center size x = -23.987 Å, y = 4.634 Å, z = = -6.149 Å, exhaustiveness = 20), and complexes with the best value of scoring functions were selected.

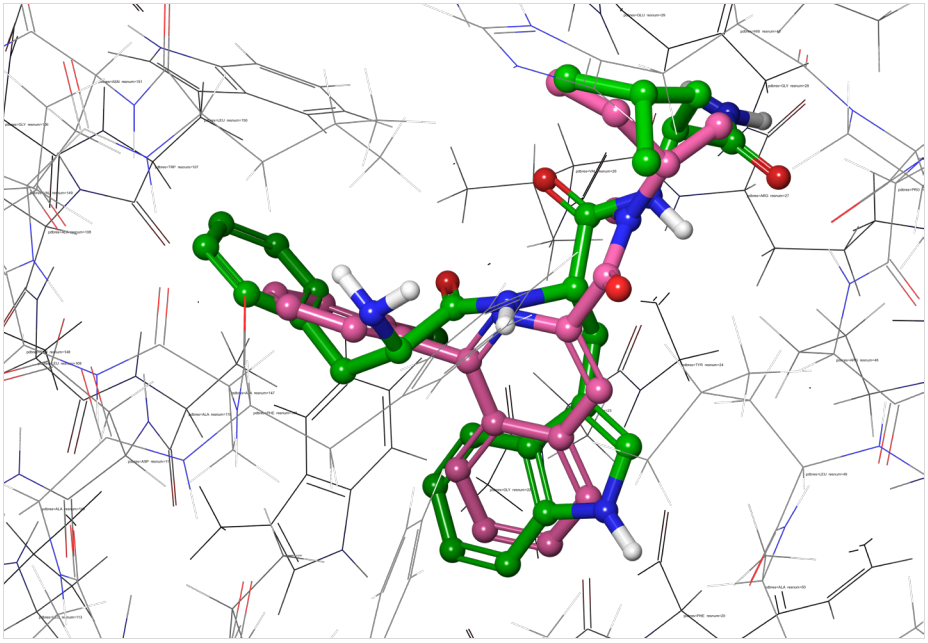


Figure S1 Location of FWL-NH_2 (in green) and PK11195 (in pink) in the PK11195 binding site in TPSO as predicted by molecular docking using in Glide 2022-4 software (visualization in Maestro 13.5).

For molecular dynamics simulations the starting structure of the TPSO complex with compound FWL-NH_2 was obtained by means of molecular docking in AutoDock Vina 1.1.2.

Molecular dynamics simulations were performed as described in[2] using the CHARMM36/CGenFF 4.4 force field[S4,S5] in GROMACS 2021.2 software[6]. For the analysis and visualization of the results, the CPPTRAJ software 5.1.0[S7] in the Amber-Tools 21 package[S8] and UCSF Chimera software 1.16[S9] were used.

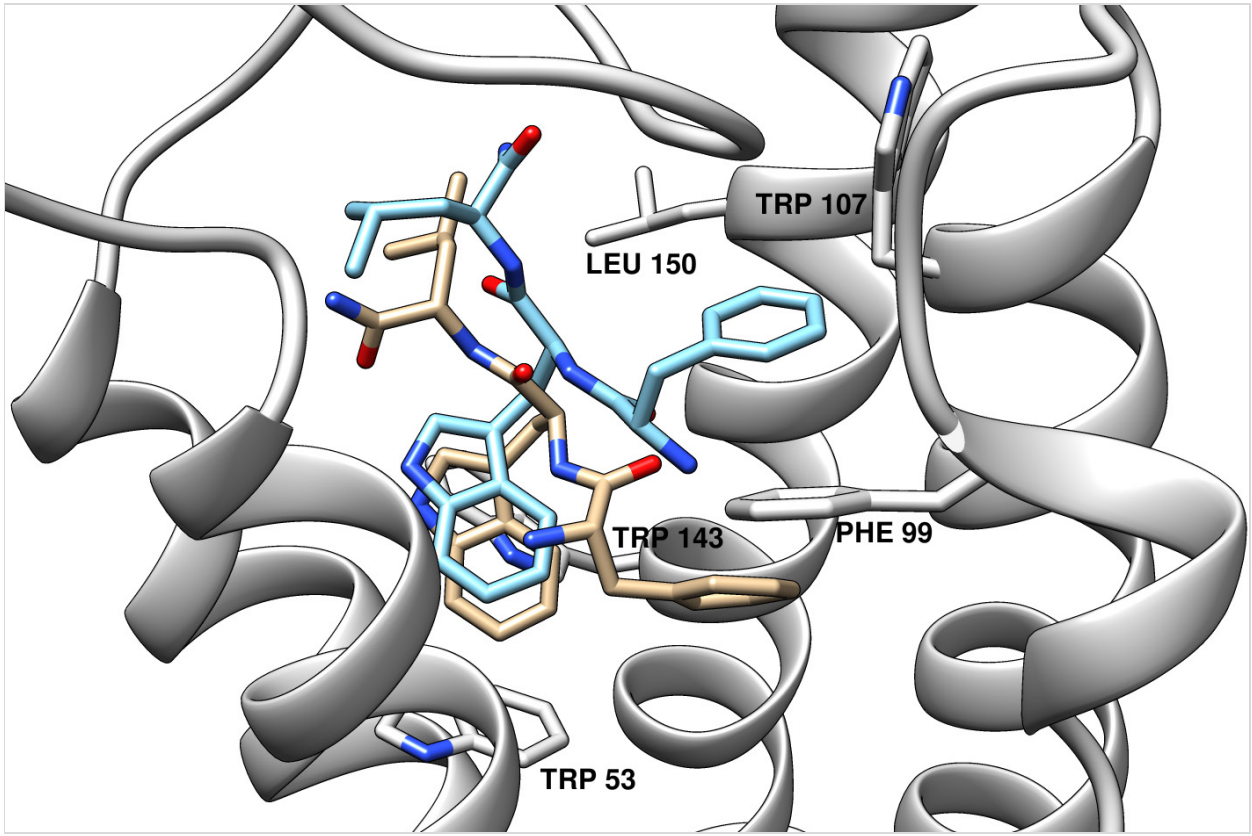


Figure S2 Comparative location of tripeptide FWL-NH_2 in the PK11195 binding site in TPSO as predicted by molecular docking using Autodock Vina 1.1.2 (represented by a light blue-colored stick model) and the binding mode of tripeptide FWL-NH_2 obtained after the molecular dynamics simulation (shown by beige stick model). The lipid molecules and hydrogen atoms are omitted for clarity.

The position of the tryptophane–leucine backbone in the binding mode of tripeptide FWL-NH_2 obtained after the molecular dynamics simulation remained virtually unchanged compared to the one obtained by molecular docking (aromatic moieties give the key interactions with amino acid residues Trp 143 and Trp 107 and Leu side chain is located near side chain of Leu 150 in TPSO).

The shift of the phenylalanine residue after the molecular dynamics simulation is clearly visible as well as the possibility of π–π stacking of its benzene ring in the new position with Phe 99 in the protein.

2. Chemistry

General information

NMR spectra were recorded on the Bruker Fourier 300 HD (Bruker Corporation, Germany, 300 and 75 MHz for ^{1}H- and ^{13}C) at room temperature; chemical shifts (δ) were measured relative to tetramethylsilan (0 ppm), DMSO-d_6 (2.50 ppm for ^{1}H). Chemical shifts (δ) are given in ppm; J values are given in Hz. Signal symbols: s – singlet, d – doublet, t – triplet, m – multiplet, br – broadened signal. When necessary, assignments of signals in NMR spectra were made using 2D techniques.

High-resolution mass spectra (HRMS) were performed using electrospray ionization (ESI). The conditions of mass spectrometric detection were developed using a TSQ Altis mass spectrometer (triple quadrupole, serial No. TSQ-A-10461, ThermoFisher Scientific, USA) using a syringe pump (flow = 10 μL/min, GD-186 solution in 1% DMSO/0.1% formic acid in acetonitrile/0.1% formic acid in water (by volume), concentration – 20 μg/mL).

Thin-layer chromatography (TLC) was performed on the aluminum silica gel plates DC Kieselgel 60 G/F254 (Merck, Germany) in solvent system: $CHCl_3$/MeOH = 9:1; amino-containing compounds were detected by ninhydrin, compounds with amide groups using chlorine and *o*-toluidine, aromatic groups were detected using UV-lamp. Analytical RP HPLC was performed using the KNAUER system (Germany). The detection was with the wavelength of 220 nm. The analysis was carried out at room temperature. The steel column 250×4.0 mm Diasfer-110-C16, 5 μm (BioChemMack) was used. The volume of the loop was 20 μl. The mobile phase A (0.5 ml TFA in a solution of 50 ml of CH_3CN + 950 ml of water) and the mobile phase B (0.05% TFA in CH_3CN) were used. Gradient elution mode was used (from 100% phase A to 100% phase B, 35 min). Flow rate was 0.9 ml min^{-1}.

Melting points were determined on the Optimelt MPA100 device (Stanford Research Systems, USA) in open capillaries without correction. Specific optical rotation was recorded on the automatic polarimeter ADP 440 (Bellingham+Stanley Ltd., Great Britain).

The starting compounds, such as protected amino acids, amides and activated succinimide esters were prepared as described in[S1]. **Cbz-L-Phe-OH** was obtained using the technique described in[S10].

Cbz-L-Trp-L-Leu-NH_2. 3.30 g (13.52 mmol) of TFA*[H-L-LeuNH_2] was dissolved in 30 ml of DMF with the addition of 2.35 ml (13.52 mmol) of DIPEA. This mixture was stirred for 0.5 h. Then 7.06 g (16.22 mmol, 20% excess) of Cbz-L-Trp-OSu in 30 ml of DMF was added. The mixture was stirred for 12 h at room temperature. The solvent was evaporated, the resulting fluent orange oil was dissolved in 200 ml of ethyl acetate, washed with 3% H_2SO_4 (2x100 ml), then 5% $NaHCO_3$ (2x100 ml) and distilled water (1x100 ml), the organic fraction was dried over

anhydrous Na_2SO_4 for 0.5 h, filtered off, the ethylacetate was evaporated. The resulting oil was dissolved in a minimum amount of DMF, diluted with 200 ml of distilled water to obtain white precipitate. The precipitate was maintained for 12 h at +5°C, filtered off, washed with distilled water and hexane and dried in vacuo over $CaCl_2$ and paraffin. The yield was 5.78 g (94%), white powder with mp 166-169°C; $[\alpha]_D^{26}$ = -31° (c 1, DMF). ^{1}H-NMR (DMSO-d_6) δ, ppm: 0.82-0.88 (m, 6H, 2 $C^{\delta}H_3$ Leu), 1.08-1.22 (m, 1H, $C^{\gamma}H_2$ Leu), 1.45 (m, 2H, $C^{\beta}H_2$ Leu), 2.90 - 3.09 (m, 2H, $C^{\beta}H_2$ Trp), 4.28-4.31 (m, 2H, $C^{\alpha}H$ Leu and $C^{\alpha}H$ Trp), 4.93 (m, 2H, $C\underline{H_2}CO$), 6.99-7.30 (m, 10H, Ar), 7.25 and 7.33 (2s, 2H, NH_2), 7.62 (d, J = 7.53 Hz, 1H, NH Leu), 7.97 (d, J = 7.83 Hz, 1H, NH Trp), 10.81 (s, 1H, NH indole).

H-L-Trp-L-Leu-NH$_2$. Through the stirring suspension of 5.78 g (12.8 mmol) of Cbz-L-Trp-L-Leu-NH_2 and 0.500 g of 10% Pd/C in 50 ml of methanol a stream of hydrogen was passed for 2 h. After reaction complete (TLC control), the catalyst was filtered, and washed with methanol. The methanol was evaporated *in vacuo*. The product was obtained in the amount of 4.71 g (99%) as a gray foam without a clear melting point, $[\alpha]_D^{26}$ = -25° (*c* 1, DMF). ^{1}H-NMR (DMSO-d_6) δ, ppm: 0.82-0.88 (2 dd, *J* = 12.95 Hz and *J* = 12.85 Hz, 6H, 2 $C^{\delta}H_3$ Leu), 1.09-1.26 (m, 1H, $C^{\gamma}H_2$ Leu), 1.45 (m, 2H, $C^{\beta}H_2$ Leu), 2.90 - 3.08 (m, 2H, $C^{\beta}H_2$ Trp), 3.50 (m, 2H, $C^{\alpha}H$ Trp), 4.14 (m, 1H, $C^{\alpha}H$ Leu), 6.94-7.55 (m, 5H, Ar), 7.05 and 7.41 (2 s, 2H, NH_2 amide), 7.60 (d, J = 7.83 Hz 1H, NH Leu), 8.05 (d, 1H, J = 8.29 Hz, NH Trp), 10.85 (broad s, 1H, NH indole).

***N*-(Benzyloxycarbonyl)phenylalanyl-tryptophanyl-leucinamide, Z-L-Phe-L-Trp-L-Leu-NH$_2$.** To the solution of 1.52 g (5 mmol) H-L-Trp-L-Leu-NH_2 in 30 ml of DMF, the 1.97 g (5.1 mmol, 20% excess) of Cbz-L-Phe-OSu was added. The reaction was carried out for 12 h at room temperature with stirring. Then 0.32 ml of DMAPA was added to neutralize the excess succinimide ester, stirred for 0.5 h. The solvent was evaporated. The resulting creamy oil was diluted with 300 ml of saturated aqueous NaCl solution to get precipitate. The resulting product was kept at +5˚C for 12 h. The precipitate was filtered off, washed with hexane, diethyl ether and dried *in vacuo* over Na_2SO_4. The product was obtained in the form of a yellow powder which was further purified by recrystallization with ethyl acetate and hexane. The yield was 1.695 g (57%), m. p. 205-209˚C, $[\alpha]_D^{23}$ -23.2˚ (*c* 1, DMF). ^{1}H-NMR (DMSO-d_6) δ, ppm: 0.85-0.88 (6H, 2 dd, 2$C^{\delta}H_3$ Leu), 1.06-1.23 (1H, m, $C^{\gamma}H_2$ Leu), 1.47-1.61 (2H, m, $C^{\beta}H_2$ Leu), 2.91 and 3.09 (2H, two d.d., $C^{\beta}H_2$ Trp), 4.27-4.60 (3H, 3 m, $C^{\alpha}H$ Leu and $C^{\alpha}H$ Trp and $C^{\alpha}H$ Phe), 4.90 (2H, m, CH_2CO), 6.99-7.25 (15 H, m, Ar), 7.25 and 7.33 (2H, two s, NH_2 amide), 7.95 (1H, d, NH Trp), 8.19 (1H, d, NH Leu), 10.82 (1H, s, NH indole)

Phenylalanyl-tryptophanyl-leucinamide, H-L-Phe-L-Trp-L-Leu-NH$_2$ (FWL-NH$_2$). 1.65 g (2.7 mol) of Cbz-L-Trp-L-Leu-NH_2 was suspended in 50 ml of methanol, then the suspension of 0.25 g of 10% Pd/C with 10 ml of methanol and 3 drops of water were accurately added. The

reaction mass was degasified using vacuum pump and filled with hydrogen from a gasholder; the procedure was repeated three times, then the reaction mixture was stirred for 5 h under hydrogen. At the end of the reaction the catalyst was filtered through a fine-pored glass filter, washed with 30 ml of methanol, and evaporated. The product was obtained in an amount of 1.59 g (99%) in the form of beige foam.

This substance was purified using column chromatography. The column with length of 7 cm and diameter of 3 cm was used. The systems were passed through: 100% of dichloromethane to 85/15 dichloromethane/methanol. Fractions of 7 ml were collected in the vials. Based on the results of TLC control, the fractions containing the target compound with R_f = 0.26 were combined and evaporated. The yield of pure product was 0.70 g in the form of white powder, m.p. 192-194˚C, $[\alpha]_D^{26}$ -26.1˚ (c 1, DMF), τ = 13.17 min.

At ionization by electrospray (H-ESI), as a result of scanning in the total ion current registration mode (Q1 mode), the molecular ion of GD-186 – $[M+H]^+$=464.4 m/z was determined, registration of positive ions. When registering negative ions, the molecular ion of the tested compound was determined worse. Scanning in the Product Ion mode (Q3) (registration of positive ions, Precursor – 464.4 m/z) revealed the main ways of fragmentation of the studied molecule and specific product ion: 334.1 m/z (447.3 m/z is loss of 17 Da – NH_3).

^{1}H-NMR (DMSO-d_6) δ, ppm: 0.68-0.98 (6H, 2 dd, 2C$^{\delta}$H$_3$ Leu), 1.31-1.52 (2H, m, C$^{\beta}$H$_2$ Leu), 1.52-1.62 (1H, m, C$^{\gamma}$H$_2$ Leu), 1.62-1.88 (2H, broad s, NH_2 Phe), 2.36-2.56 and 2.76-2.94 (2H, two dd, C$^{\beta}$H$_2$ Phe), 2.94-3.24 (2H, m, C$^{\beta}$H$_2$ Trp), 3.39 (1 H, m, C$^{\alpha}$H Phe), 4.27 (1H, m, C$^{\alpha}$H Leu), 4.62 (1H, m, C$^{\alpha}$H Trp), 6.96 - 7.90 (12H, m, Ar, NH_2 amide), 7.62 (1H, d, NH Leu), 8.03 (1H, d, NH Trp), 10.89 (1 H, s, NH indole).

^{13}C-NMR (DMSO-d_6) δ, ppm: 22.12 and 23.50 (2C, 2C$^{\delta}$H$_3$ Leu), 24.67 (1C, C$^{\gamma}$H$_2$ Leu), 28.30 (1C, C$^{\beta}$H$_2$ Trp), 40.96 (1C, C$^{\beta}$H$_2$ Leu), 41.50 (1C, C$^{\beta}$H$_2$ Phe), 51.34 (1C, C$^{\alpha}$H Leu), 53.42 (1C, C$^{\alpha}$H Trp), 56.44 (1C, C$^{\alpha}$H Phe), 110.13 (1C, C^{9} Trp), 111.69 (1C, C^{15} Trp), 118.66 (1C, C^{14} Trp), 119.05 (1C, C^{12} Trp), 121.27 (1C, C^{13} Trp), 124.17 (1C, C^{10} Trp), 126.57 (1C, C^{23} Phe), 127.98 (1C, C^{11} Trp), 128.56 (1C, C^{22} Phe), 129.75 (1C, C^{21} Phe), 136.51(1C, C^{16} Trp), 139.03 (1C, C^{20} Phe), 171.50 (1C, C=O Trp), 174.51 (2C, C=O Leu and C=O Phe).

Figure S3 ^{1}H-NMR of L-Phe-L-Trp-L-Leu-NH_2

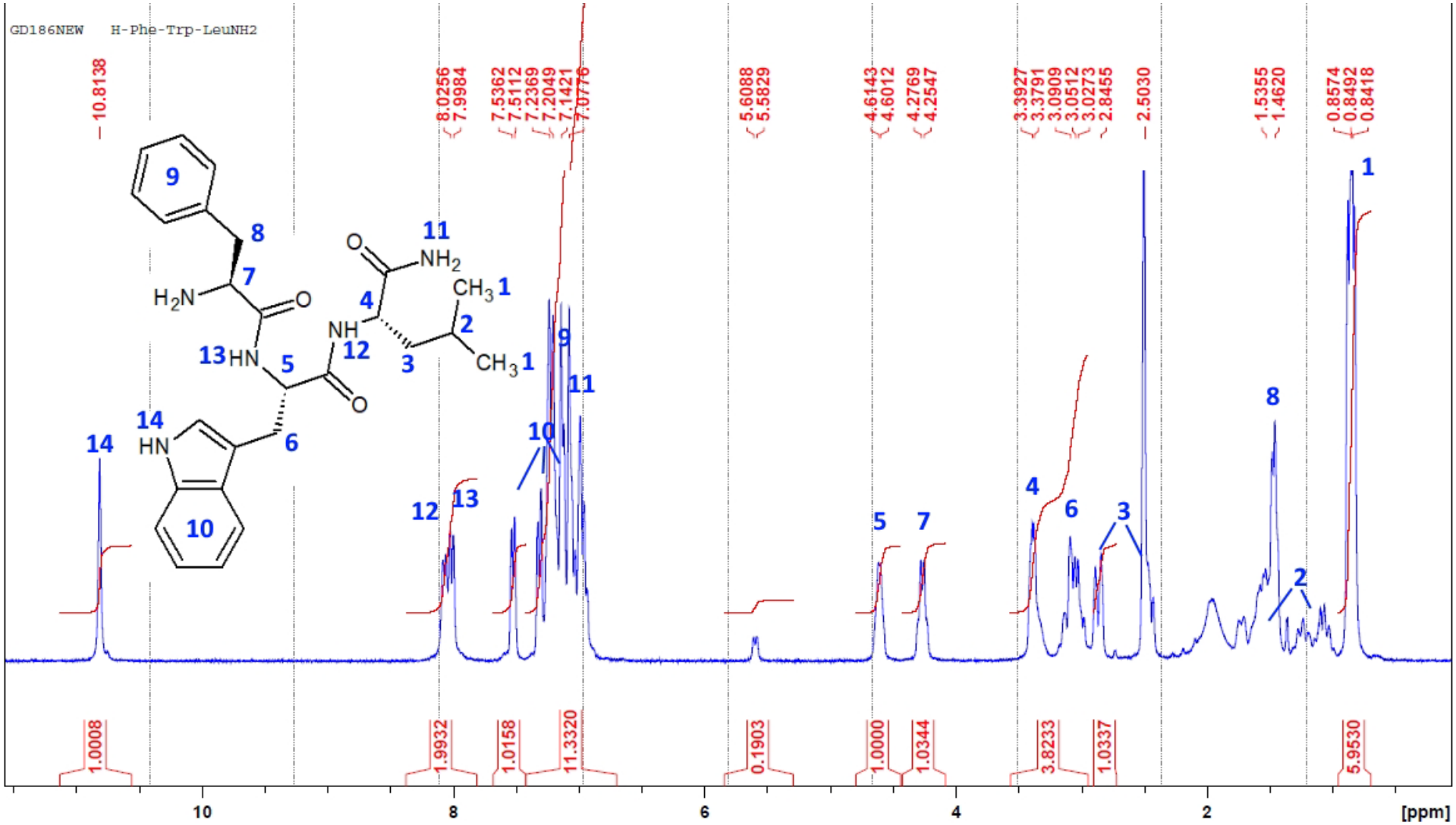


Figure S4 ^{13}C-NMR of L-Phe-L-Trp-L-Leu-NH_2

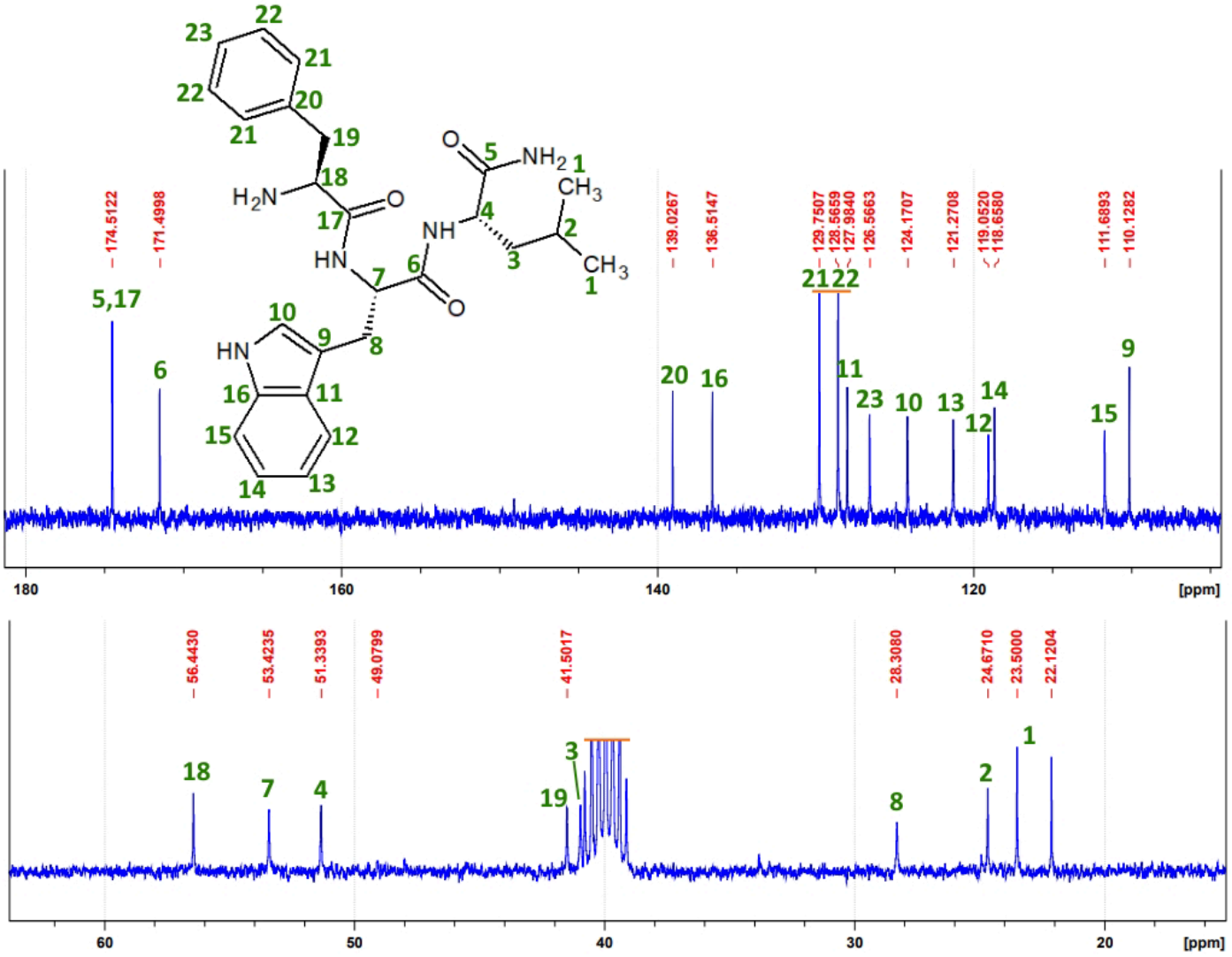
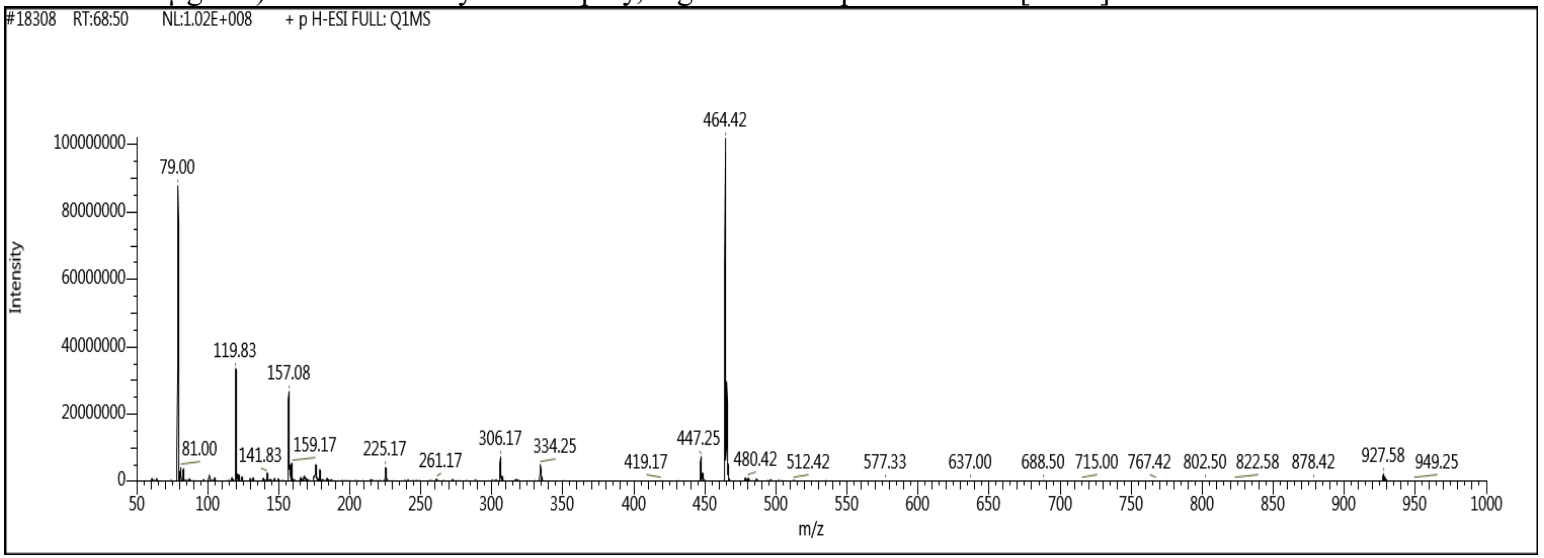
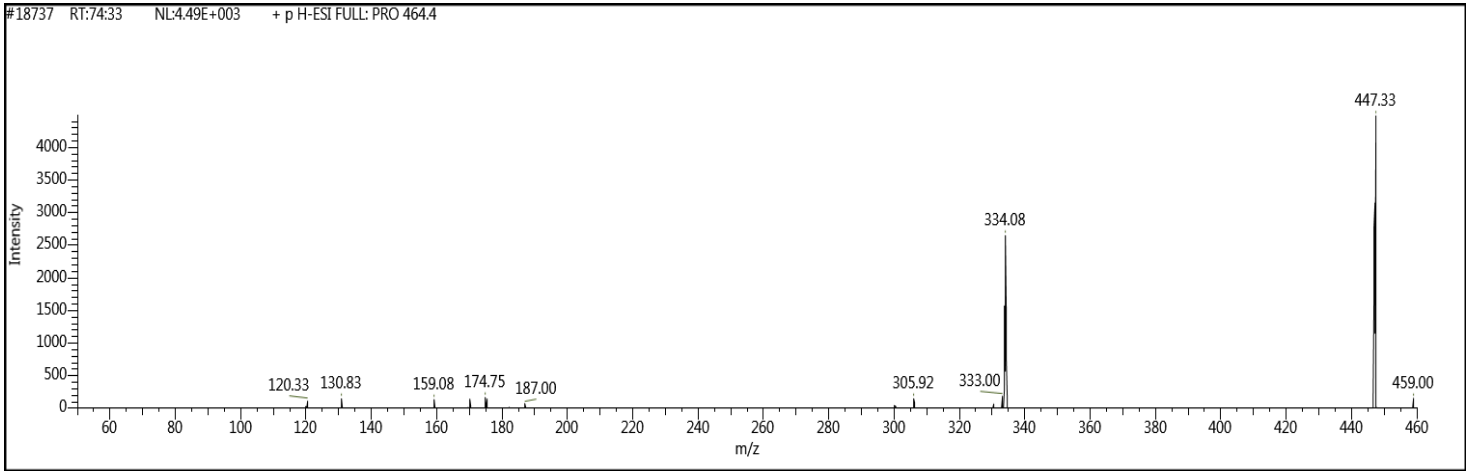


Figure S5 Mass spectra of FWL-NH_2

(Q1 mode, total ion current) of FWL-NH_2 (FWL-NH_2 solution in 1%DMSO/0.1% formic acid in acetonitrile/0.1% formic acid in water (by volume), concentration – 20 µg/mL) at ionization by electrospray, registration of positive ions. $[M+H]^+$ – 464.4 m/z.



Mass spectrum (Product Ion mode) of FWL-NH_2 (FWL-NH_2 solution in 1%DMSO/0.1% formic acid in acetonitrile/0.1% formic acid in water (by volume), concentration – 20 µg/mL) at ionization by electrospray, registration of positive ions. Product Ion – 334.1 (447.3 m/z is loss of 17 Da – NH_3).



3. *In vivo* studies

Animals

Adult male Balb/c mice and ICR mice weighing 19 - 26 g were used in this study. The animals were obtained from the Branch "Andreevka" of the Federal State Budgetary Institution of Science "Scientific Center for Biomedical Technologies" of the Federal Medical and Biological Agency (Moscow region, Russia). Mice were adapted for 2 weeks prior to testing in our Institute's vivarium at the environmentally controlled properties: 12 h light-dark cycle (lights on at 08:00 o'clock), the temperature of 22˚C ± 2˚C and the humidity of 60 ± 10%. All the mice were put on a standard diet with food and water available ad libitum. The animals were split in groups of 8 according to body weight with the deviation from the mean value of no more than ± 10%. All animals were kept for 24 h in the experimental room at "home" cages before experiments.

Ethical approval

The *in vivo* experiments involving animals were conducted in accordance with international regulations (Directive 2010/63/EU of the European Parliament and of the Council of the European Union of September 22, 2010, on the protection of animals used for scientific purposes). The study was approved by the Biomedical Ethics Committee of the V.V. Zakusov Research Institute of Pharmacology (protocol no. 2, January 30, 2023).

Elevated plus maze test

The anxiolytic effect of compound was evaluated in the elevated plus-maze (EPM) test, which is widely used to identify compounds with this type of activity[11]. The EPM setup ("Open Science", Moscow, Russia) was used in its basic configuration: the open and enclosed arms were 30 cm in length and 5 cm in width, with the enclosed arms featuring walls of 15 cm in height. The cross-shaped arena was constructed from gray opaque plastic and elevated 40 cm above the floor. The dipeptide GD-102[1] was used as a positive control at the dose of 0.5 mg kg^{-1}. Animals were carefully placed in the center of the EPM in a random direction relative to the open and closed arms. The test was carried out in low light (11-15 lx in open arms and about 2-5 lx in closed arms). The behavior of the animals was recorded using a video camera. Registration time was 4 minutes. Video data processing was carried out using the RealTimer program ("Open Science", Moscow, Russia). The number of entries into open and enclosed arms, as well as the time spent in open and enclosed arms, were documented[12]. The indicators "time in open arms" and "number of entries into open arms" were calculated in relative units (in %) using the formulas:

time in open arms, % = (time in open arms in s / total time in s) × 100%;

number of entries into open arms, % = (number of entries into open arms/total number of

entries into open and closed arms) ×100%.

Table S1. Anxiolytic activity of FWL-NH_2 in the elevated plus maze test in BALB/c mice.

Group	Time spent in open arms, s	Time spent in closed arms, s	Number of open arms entries	Number of closed arms entries	Relative time spent in open arms, %	Relative number of open arms entries, %
Control	2.7±1.1	184.0±11.7	0.8±0.3	6.6±1.1	1.6±0.6	12.4±4.35
FWL-NH_2 (1 mg kg^{-1})	5.3±2.2	178.0±16.2	0.9±0.3	6.2±1.1	3.0±1.20	12.1±3.6
FWL-NH_2 (5 mg kg^{-1})	33.7±25.6	146.3±25.8	1.3±0.5	4.3±1.1	16.3±10.7	23.9±10.7
FWL-NH_2 (10 mg/kg)	38.2±16.7[c] (p=0.067)	143.4±22.4	2.3±0.7	4.5±0.7	21.5±9.0[c] (p=0.0761)	32.1±4.8[a] (p=0.0399)
GD-102 (0.5 mg kg^{-1})	48.64±9.937[a] (0.0016)	116.9±16.03 * (p=0.047)	3.10±0.53[a] (p=0.0106)	5.50±0.75	31.2±6.06[b] (p=0.0018)	34.50±4.87[b] (p=0.0088)

[a] $p<0.05$; [b] $p<0.01$ – compared with control (Kruskal-Wallace test followed by intergroup pairwise comparison using Dunn's test); [c] Trend ($p<0.10$) compared with control (Kruskal-Wallace test followed by intergroup pairwise comparison using Dunn's test).

Porsolt forced swimming test.

Forced swimming test in mice[S13] was performed using a modified two-session swim technique[S14]. The test setup ("Open Science", Moscow, Russia) consisted of 5 cylinders made of transparent plastic, diameter of 10 cm, height of 30 cm. The cylinders were filled with water at 22-23°C to 2/3 of the height - the level at which animals do not have the opportunity to rest on the bottom of the cylinder with their paws or tail. Animals were placed in cylinders with water for 10 minutes. After 24 h, the test session was carried out - the animals were re-placed in the same conditions for 5 min. The behavior of the animals was recorded using a video camera. Video data processing was carried out using the RealTimer program ("Open Science", Moscow, Russia). The total time the animals maintained the immobility pose was recorded. A decrease in the total time of immobility was considered as indicator of antidepressant-like activity. FWL-NH_2 was injected as suspension with Tween-80 in distilled water (one drop per 5 ml of water). Amitriptyline (10 mg kg^{-1}, *i.p.*) was used as a reference drug. Control animals received a 1% solution of Tween 80. The volume of intravenous administration was 10 ml per 1 kg of body weight of mice. The mice were randomly divided into 4 groups:

1. "FWL-NH_2, 10 mg kg^{-1}" (n=9)
2. "FWL-NH_2, 30 mg kg^{-1}" (n=10)
3. "Amitriptyline, 10 mg kg^{-1}" (n=9)
4. "Control" (n=11)

Compound FWL-NH_2 was administered to mice 30 min before the test session, and Amitriptyline 1 h before test session in cylinders with water. Animals from the control group

were injected with a 1% solution of Tween 80 1 h before the testing. Data were tested for normality using the Shapiro-Wilk W test. Since the data distribution was not normal in all experimental groups, the nonparametric Kruskal-Wallis test was used to identify intergroup differences, followed by pairwise intergroup comparisons using Dunn's test. Differences were considered statistically significant at $p<0.05$.

Table S2. Antidepressant-like effect of a single i.p. injection of FWL-NH_2 in the forced swimming test in BALB/c mice.

Dose	Average immobility time, sec	Immobility time, % of control group
Control	183.8±8.8	100
FWL-NH_2, 10 mg kg^{-1}	135.4±15.4*	73*
FWL-NH_2, 30 mg kg^{-1}	125.3±7.2**	68**
Amitriptyline, 10 mg kg^{-1}	90.3±16.4****	49****

* - $p<0.05$; ** - $p<0.01$, **** - $p<0.0005$ – compared with control (Kruskal-Wallace test followed by intergroup pairwise comparison using Dunn's test)

4. *In vitro* studies

Materials and drugs. The study was carried out using immortalized HT-22 mouse hippocampal cells. All manipulations with cells were performed in strictly sterile conditions. For the experiment, cells were seeded into 96-wells culture plates treated with poly-D-lysine (Corning, USA) at a density of 3500 per well and incubated in DMEM medium (HyClone, USA) containing 2 mM L-glutamine (MP Bioscience , USA) and 5% fetal bovine serum (FBS, ThermoFisher, USA) at 37°C in an atmosphere containing 5% CO_2 until a monolayer is formed. Oxidative stress was reproduced by adding H_2O_2 to the culture medium ("Obnovleniye", Russia) in a final concentration of 1.5 mM for 30 minutes at 37°C as discribed in[S15]. Next, the culture medium containing H_2O_2 replaced with a normal one and after 4 hours cell viability was determined using the MTT test.

Cell viability assessment (MTT-test). At the end of the experiment, the culture medium was replaced with an MTT solution (0.5 mg ml^{-1}) and incubated for 30 min at 37°C[S16]. The MTT solution was then removed from the wells and DMSO was added to dissolve the formazan. After 15 min, light absorption was measured on a Multiscan spectrophotometer ("Thermo") at a wavelength of 600 nm.

FWL-NH_2 was dissolved in DMSO. Next, a series of serial dilutions were prepared in deionized water to get 2% DMSO. Also, 2% DMSO solution was added to the control. FWL-NH_2 was added 24 h before and immediately after cell damage in final concentrations from 10^{-5} to 10^{-8}M.

Processing MTT test data. Statistical processing of the data was carried out using one-way analysis of variance ANOVA and the Kruskal-Wallis test followed by Dunn's test.

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