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Novel Proline Transporter Inhibitor (LQFM215) Presents Antipsychotic Effect in Ketamine Model of Schizophrenia

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Abstract

The glutamatergic hypothesis of schizophrenia suggests a correlation between NMDA receptor hypofunction and negative psychotic symptoms. It has been observed that the expression of the proline transporter (PROT) in the central nervous system (CNS) is associated with glutamatergic neurotransmission, as L-proline has the capacity to activate and modulate AMPA and NMDA receptors. In this study, we aimed to investigate whether inhibition of proline transporters could enhance glutamatergic neurotransmission and potentially exhibit antipsychotic effects in an experimental schizophrenia model. Using molecular dynamics analysis in silico, we validated an innovative PROT inhibitor, LQFM215. We quantified the cytotoxicity of LQFM215 in the Lund human mesencephalic cell line (LUHMES). Subsequently, we employed the ketamine-induced psychosis model to evaluate the antipsychotic potential of the inhibitor, employing behavioral tests including open-field, three-chamber interaction, and prepulse inhibition (PPI). Our results demonstrate that LQFM215, at pharmacologically active concentrations, exhibited negligible neurotoxicity when astrocytes were co-cultured with neurons. In the ketamine-induced psychosis model, LQFM215 effectively reduced hyperlocomotion and enhanced social interaction in a three-chamber social approach task across all administered doses. Moreover, the compound successfully prevented the ketamine-induced disruption of sensorimotor gating in the PPI test at all tested doses. Overall, these findings suggest that PROT inhibition could serve as a potential therapeutic target for managing symptoms of schizophrenia model.

Keywords Schizophrenia · Glutamatergic neurotransmission · Drug discovery · Target validation · SLC family transporters · SLC6A7

Abbreviations

AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AU	Arbitrary units
CEUA	Animal Ethics Committee
CNS	Central nervous system
DMEM/F12	Dulbecco's modified Eagle's medium/Ham's nutrient mixture F-12

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DMSO	Dimethyl sulfoxide
GDNF	Anti-glial cell-derived neurotrophic factor
GlyT1	Type 1 glycine transporters
IC50	Maximal inhibitory concentration
IP	Intraperitoneal injection
LDH	Lactate dehydrogenase
LTD	Long-term depression
LTP	Long-term potentiation
LUHMES	Lund human mesencephalic cell line
mAGES	Mouse embryonic stem cells
NFPS	N[3-(4′-fluorophenyl)-3-(4′-phenylphenoxy)propyl]sarcosine
NMDA	N-methyl-D-aspartate
NMDAR	NMDA receptors
PBS	Phosphate buffered saline
PCP	Phencyclidine
PPI	Prepulse inhibition
PRODH	Proline dehydrogenase
PROT	Proline transporter
RMSD	Root mean square deviation
SEM	Standard error of the mean
SCL6A15	Solute carrier family 6 member 15
SCL6A17	Solute carrier family 6 member 17
SCL6A20	Solute carrier family 6 member 20
SCL6A7	Solute carrier family 6 member 7

Introduction

Schizophrenia is a profound mental disorder that significantly impacts perception, cognition, and emotions. This frequently results in disruptions of reality, delusions, hallucinations, and challenges in maintaining cognitive coherence [1]. Antipsychotic medications, which normally modulate the function of dopaminergic and/or serotoninergic neurotransmissions, effectively manage positive symptoms such as hallucinations and delusions. However, addressing negative symptoms remains a formidable challenge in treatment. Negative symptoms of schizophrenia, including apathy, anhedonia, impoverished speech, and lack of motivation, may stem from problems within neural circuits involving the glutamatergic system [1]. It is postulated that these problems intricately affect synaptic plasticity, inter-neuronal communication, and the nuanced regulation of affective and cognitive processes. Given this, a strategic exploration of novel targets capable of modulating glutamatergic neurotransmission, coupled with the discovery of new compounds targeting negative symptoms, is an imperative endeavor to enhance schizophrenia therapy.

Animal models play a crucial role in the study of schizophrenia symptoms, particularly negative and cognitive symptoms. Therefore, it is necessary to identify specific behavioral tasks in rodents that are relevant and have translational significance for the specific cognitive domains affected in schizophrenia [2]. Some notable tasks include the novel object recognition task, social interaction test, pre-pulse inhibition (PPI) of the acoustic startle response, and the Morris water maze [3]. Numerous models using N-methyl-D-aspartate receptor (NMDAR) antagonists such as ketamine, MK-801 and phencyclidine (PCP) mimic the negative symptoms and cognitive deficits of schizophrenia. These antagonists, in sub chronic approaches for example, can induce social withdrawal in animals [4].

NMDAR is an ionotropic receptor triggered by glutamate and its co-agonists (glycine and serine), facilitating the influx of Na^+ and Ca^{2+} into neurons [5]. NMDARs are made up of two subunits: GluN1, which contains the co-agonist binding site, and GluN2, which contains the glutamate and PSD-95 binding sites. Long-term potentiation (LTP) requires GluN2A-containing NMDAR activation, whereas long-term depression (LTD) requires GluN2B-containing NMDAR activation [5]. Regulating NMDAR activation is vital for neuronal function, and the upregulation of these receptors could prove beneficial for schizophrenia treatment [6, 7].

The pharmacological application of L-proline to hippocampal slices induces neuronal depolarization through the direct activation of NMDAR and AMPA receptors [8, 9]. As of now, there isn't a specific receptor for proline identified. However, several transporters, such as PROT (SLC6A7), B0AT2 (SLC6A15), NTT4 (SLC6A17), and IMINO (SLC6A20), are found in cerebral tissue. PROT, a brain-specific member of the Slc6 transporter family, has a high affinity for L-proline and plays a pivotal role in regulating neurotransmitter signaling as well as maintaining brain homeostasis [10–12]. It's postulated that PROT modulates the spatial and temporal distribution of L-proline at glutamatergic synapses due to its rapid uptake into presynaptic terminals. Nevertheless, a full understanding of its role in this dynamic process is still being elucidated.

It is noteworthy that the biosynthesis of L-proline is closely associated with the biochemical cascade facilitating glutamate production [10–12]. Within mitochondrial processes, the Krebs cycle enzymatically transforms α-ketoglutarate into glutamate. This conversion is then succeeded by the enzymatic action of P5C synthase, which converts glutamate into γ-semialdehyde-glutamate. This intermediate, in turn, spontaneously cyclizes to form P5C [13]. In the cytosol, enzymatic reduction of P5C culminates in the synthesis of L-proline. On the flip side, L-proline degradation occurs within the mitochondria, mediated by the proline oxidase to form P5C. This catabolic route produces intermediates that stand ready for potential reentry into glutamate metabolic pathway [13].

Given the neuroanatomical distribution of PROT and the ability of L-Proline to activate NMDAR, a sophisticated

mechanism of neuromodulation in glutamatergic transmission is implied. As such, blocking PROT may increase proline concentration in the synapse and potentiate NMDAR. Exploring the pharmacological potential of PROT inhibition may reveal new therapeutic applications for neurological and psychiatric disorders. In this study, we describe the pharmacological evaluation of the compound LQFM215, a new PROT inhibitor, and its effect in an animal model of schizophrenia.

Materials and Methods

Animals

Behavioral tests were performed using male Swiss mice, aged 8 to 12 weeks, and weighing between 40 and 50 g. Our animal studies comply with the ARRIVE guidelines [14], and the animals were randomly selected for the experiment. These animals were provided with standard feed and water ad libitum and maintained under temperature-controlled conditions on a 12 h artificial light/dark cycle (6am to 6pm). They were housed in plastic cages (50 × 35 × 15 cm), with each cage accommodating ten mice. The animals were sourced from the animal facilities of the Federal University of Goiás (UFG) and the University of São Paulo—Ribeirão Preto (USP), and all experimental protocols received approval from the Animal Ethics Committee at UFG (CEUA Protocol: 44/2018) and USP (CEUA Protocol: 0008/2021R1).

Bioinformatics: Molecular Dynamics

Based on the results of previous studies on the modelling of the PROT and molecular docking with the compound LQFM215, we conducted molecular dynamics analysis to verify the dynamics of protein-ligand interaction and the stability of the complex [15]. The docking-derived complex was submitted to the CHARMM-GUI server for dynamic solution construction [16–18]. Initially, we constructed an octahedral water box with a 10Å diameter, followed by system equilibration using 0.15 M NaCl (ions were introduced during the molecular equilibrium stage) at a temperature of 310 K (approximately 36 °C). Upon setup completion, the server supplied scripts to execute the dynamics via NAMD on a local machine, an equilibration script for the system, and a production dynamics script that ran for a duration of 100 ns [19]. Finally, trajectory data from the molecular dynamics was generated and examined utilizing VMD 1.9.3 software [20].

In Vitro Analysis

Human Neuronal Precursor Cells: LUHMES and Astrocyte Cell Co-culture

LUHMES (*Lund human mesencephalic*) cells were cultured according to established protocols [21, 22]. In brief, we used cell culture flasks and plates procured from Sarstedt and coated them with a water-based solution of poly-L-ornithine (50 µg/mL) and fibronectin (1 µg/mL) from Sigma. LUHMES were sustained in Advanced Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F-12 (DMEM/F12) (Invitrogen) supplemented with 1× N2 supplement (Invitrogen), 2 mM L-glutamine (Gibco), and 40 ng/mL recombinant basic fibroblast growth factor (R+D Systems). Differentiation was instigated by introducing a differentiation medium that included Advanced DMEM/F12 with 1× N2 supplement (Invitrogen), 2 mM L-glutamine (Gibco), 1 mM dbcAMP (Sigma), 2 µM tetracycline (Sigma), and 2 ng/mL Anti-Glial Cell-derived Neurotrophic Factor (Anti-GDNF) (R+D Systems). On day 2 or 5 of differentiation, cells were detached using 0.05% trypsin/EDTA (Invitrogen), placed in 96-well plates (100,000 cells/cm) and treated with compounds (100 to 0.39 µM) for a 24 h duration. Neurite area and cell viability assessments were conducted 22–26 h post-treatment initiation, utilizing image acquisition. The final dimethyl sulfoxide (DMSO) concentration was consistently 0.1% (V/V) and served as a solvent control. Quantification of the neurite area and cell viability was achieved through high content imaging, as previously outlined [23].

Co-culture experiments involving LUHMES and astrocytes cells adhered to the methodology outlined by Gutbier et al. (2018). In summary, mouse embryonic stem cells (mAGES) were differentiated into mouse astrocytes using N2B27 medium supplemented with 20 ng/mL BMP4 (R+D Systems) over a 3 day period (100,000 cells/cm). Subsequently, day 2 pre-differentiated LUHMES (100,000 cells/cm) were seeded onto mAGES-derived astrocytes in LUHMES differentiation medium for an additional 3 days. These cells underwent a 24 h treatment with a two-fold serial dilution of LQFM215 (100 to 0.39 µM), and cell viability was evaluated through lactate dehydrogenase (LDH) release.

Viability and Neurite Area Assessments

Cells were stained using Hoechst H-33,342 (1 µg/mL) and calcein-AM (1 µM) to facilitate image acquisition [21]. The neurite area was calculated as the total calcein-positive area, adjusted for the somatic area. This somatic area is represented by the Hoechst-positive area, surrounded by a 3.2 µm ring representing the neuronal body. These images were also utilized to assess cell viability. Cells that were double-positive were classified as viable, while Hoechst-positive objects

lacking calcein stain were counted as dead cells. Viability was expressed as the ratio of viable cells to total cells, multiplied by 100%. At least three independent experiments were carried out, each with 3–5 technical replicates. Initially, data from the technical replicates were expressed as a percentage of the solvent-treated. Then, technical replicates were averaged to minimize technical variability. Subsequently, the mean of three biological replicates was computed for the neurite area and viability relative to the solvent control. Fits were calculated using a 4-parameter Hill model with the baseline constrained to the maximum of 100% of the solvent-treated signal. The Hill model equation was solved for $f(x) = 50\%$ to determine the EC50, signifying the concentration at which the effect on neurite growth inhibition/destabilization reached 50% of the solvent control.

LDH Release Assay

LDH release was independently identified in the supernatant and the corresponding cell homogenate [21]. The medium was moved to a separate plate, after which the cells were lysed in phosphate buffered saline (PBS)/0.1% Triton X-100 for a duration of 2 h. A 20 µL sample was combined with 180 µL of reaction buffer containing NADH (100 µM) and sodium pyruvate (600 µM) in a potassium-phosphate buffer (pH 7.4). Absorption at 340 nm was measured in 1 min intervals over a period of 15 min, all at 37 °C. The rate of NADH consumption is representative of LDH activity. Viability was expressed as the ratio of LDH activity observed in the supernatant to the total LDH activity (that includes both the supernatant and cell homogenate). The data was conveyed as a percentage of viability, with untreated cells taken as having 100% viability, and compared against various concentrations.

In Vivo Analysis

Drug Administration and Ketamine-Induced Psychosis Model

The new compound LQFM215 was synthesized as described [15] and were dissolved in a 10% DMSO (Sigma) solution and applied in physiological saline. Different doses of the LQFM215 (10 mg/kg, 20 mg/kg, 30 mg/kg) were administered by intraperitoneal injection (IP) 30 min before the experiments and the control animals received injections of the vehicle. These doses were established from the characterization of the compound in our previous work, as well as the per se effect of the compound on the behavior of the animals [15].

Ketamine-induced psychosis model (10 mice per group) was performed by single intraperitoneal administration of 50 mg/kg of ketamine (Cetamin–Syntec) approximately 30 min before behavioral tests. LQFM215 application was administrated 5 min after ketamine administration, in a second injection. The dose of ketamine and the induction of an acute model with a single dose was established according to previous studies [24–27]. Animals were randomized for treatment. Data collection and evaluation of all behavioral experiments were performed blindly of the group identity and each experiment was performed independently.

Open Field Test

The open-field test is a straightforward procedure used to evaluate locomotion, exploration, and anxiety in test subjects [10]. This test investigates a subject's inherent reactions to open spaces while considering their exploratory drive. The mice were placed in an acrylic apparatus after 1 h of habituation to the behavior room and were then subjected to the test 30 min post-drug administration. Their activities were recorded for a duration of 10 min, and the following parameters were assessed: rearing (elevation of rear foot), grooming (self-cleaning reflex), crossing (number of intersections), immobility duration, number of entries into the center, and total distance covered.

Three-Chamber Test

The three-chamber test was utilized to assess social interaction in mice subjected to a ketamine-induced psychosis model [28]. Initially, each subject was gently placed in the unoccupied three-chamber apparatus for a 10 min period of spatial exploration. Subsequently, an unfamiliar mouse was introduced into the apparatus, confined within a pencil cup in the right chamber. Simultaneously, an empty pencil cup was placed in the left chamber. The test subject was then positioned in the central chamber and allowed to explore the three-chamber apparatus for an additional 10 min. The following parameters were analyzed: time spent investigating each pencil cup, time spent in each chamber, and the number of interactions with each cup. Acute model induction with ketamine and treatment with LQFM215 were performed 30 min before the test phase. Subjects (10 animals per group) were randomly assigned to treatment groups.

Pre-pulse Inhibition Test (PPI)

PPI refers to the reduction in the startle reflex response in various mammalian species when a subthreshold stimulus precedes a startling acoustic stimulus. PPI serves as an objective metric of sensorimotor gating, and research indicates that schizophrenia patients often exhibit PPI impairments [29]. As previously detailed [30], the experiment was conducted using two identical startle response systems (Med Associates, Inc., USA). A continuous acoustic signal

maintained a 65 dB background white noise level. The pulse, which was a 110 dB white noise burst, had a rise/fall time of 5 ms and a duration of 20 ms. The pre-pulse was set at an 85-dB intensity, a 7 kHz frequency, and a 10 ms duration. The PPI test comprised 24 trials, pseudo-randomly divided into four categories, with stimuli presented at 10–30 s interstimulus intervals. These 24 trials were split into: (1) pulse alone, (2) pre-pulse alone, (3) pre-pulse+pulse, and (4) no stimulation. The startle amplitude for the pre-pulse plus pulse was expressed as a percentage of the pulse-alone startle amplitude, calculated as follows: %PPI = [100 × (mean startle of pulse-alone trials − mean startle of pre-pulse-pulse trials)/mean startle of pulse-alone trials]. The platform, set at a standard weight of 40 g, was calibrated by adjusting the gain of the load cell amplifier to 150 arbitrary units (AU). The load cell's range was set between − 2047 and + 2047 AU. Model induction with ketamine and LQFM215 treatment occurred 30 min prior to testing.

Statistical Analysis

Sample sizes were calculated assuming an α error of 0.05, power of 0.8, effect size of 0.71 for the Ketamine model analysis, and 0.94 for molecular analysis using G*Power software (3.1.9.2). The equality of variances was determined using both the Brown–Forsythe test and Bartlett's test. Data from behavioral tests were compared using one-way ANOVA, followed by Dunnett's post-test and Dunn's multiple comparison test. Statistical significance was set at $p < 0.05$. Results are reported as the mean ± standard error of the mean (SEM). All data conformed to a normal distribution and were evaluated using the Shapiro–Wilk normality test (significance level alpha = 0.05). Data analysis was performed using Graph Pad Prism, version 9.0 (GraphPad Software Inc., San Diego, CA, USA).

Results

Molecular Dynamics of the PROT-LQFM215 Complex

Root mean square deviation (RMSD) is a metric that quantifies the structural deviation between two or more structures, often employed to evaluate the stability or conformational fluctuations of a protein or protein-ligand complex over time. For this analysis, the PROT-LQFM215 complex, obtained from the docking performed earlier [15], was assessed for its average RMSD over the course of a 100 ns molecular dynamics simulation (Fig. 1A, B). The complex can be observed in Fig. 1A, immersed in a water box with balanced ions, assembled in silico through the CHARMM-GUI. Figure 1B highlights the key amino acid residues of the active site interacting with the ligand and influencing the balance of this interaction.

Low RMSD values (< 3Å) suggest structural similarity (in this case, indicating a more stable interaction), whereas high RMSD values could denote significant conformational changes. Throughout the analysis period, the RMSD maintained an average of 2.069 Å ± 0.549, peaking at 3.921 Å during the early stages of the dynamics, likely corresponding to the system's equilibration phase (Fig. 1C). These findings confirm the formation and stability of the protein-ligand complex initially generated in the docking process over a simulation duration of 100 ns, given that the average RMSD remained under 3 Å. A demonstration video of the dynamic simulation and a figure showcasing the interactions are available as supplementary material.

PROT Inhibitor Present Low Toxicity Against LUHMES Cells

The LUHMES cell line, consisting of immortalized human dopaminergic neuronal precursor cells, serves as a valuable model for evaluating the effects of chemicals on the viability and neurite growth of human dopaminergic-like neurons. Figure 2 demonstrates the impact of PROT inhibitors on neurite growth and cell viability in LUHMES cells. The viability of LUHMES cells during the neurite development phase, day 2 of differentiation, was reduced by 100 µM LQFM215 after a 24 h exposure period (Fig. 2A, B). Image analysis revealed a decrease in neurite area with LQFM-215 treatment at a concentration that results in 50% inhibition (IC50:3µM), suggesting that PROT inhibitors impede neurite formation during cell maturation. In mature neurons (d5-6), LQFM215 (IC50 = 14 µM) elicited a concentration-dependent reduction in both viability and neurite growth. It was noted that the neurite growth was affected at similar concentrations as overall cell viability in mature cultures.

We further analyzed the effect of LQFM215 on a co-culture of human LUHMES cells and astrocytes. LUHMES cells, pre-differentiated on day 2, were co-cultured with astrocytes for an additional 3 days and subsequently treated with LQFM215 for 24 h. After a 24 h treatment period, LQFM215 reduced the cell viability in day-5 LUHMES cells alone (IC50 of 9 µM). However, the cytotoxic effect of LQFM215 was mitigated in the co-culture with astrocytes (IC50 > 50 µM) (Fig. 3). This finding suggests that the cytotoxic effect of LQFM215 may be selectively targeted at neuron-like cells, and this effect can be ameliorated in a more complex cellular environment, as found in brain tissue, potentially due to protective effects offered by astrocytes.

To assess whether the impact of LQFM215 on neurite growth could be reversed, LUHMES cells were pre-differentiated in a glucose medium for 2 days and then

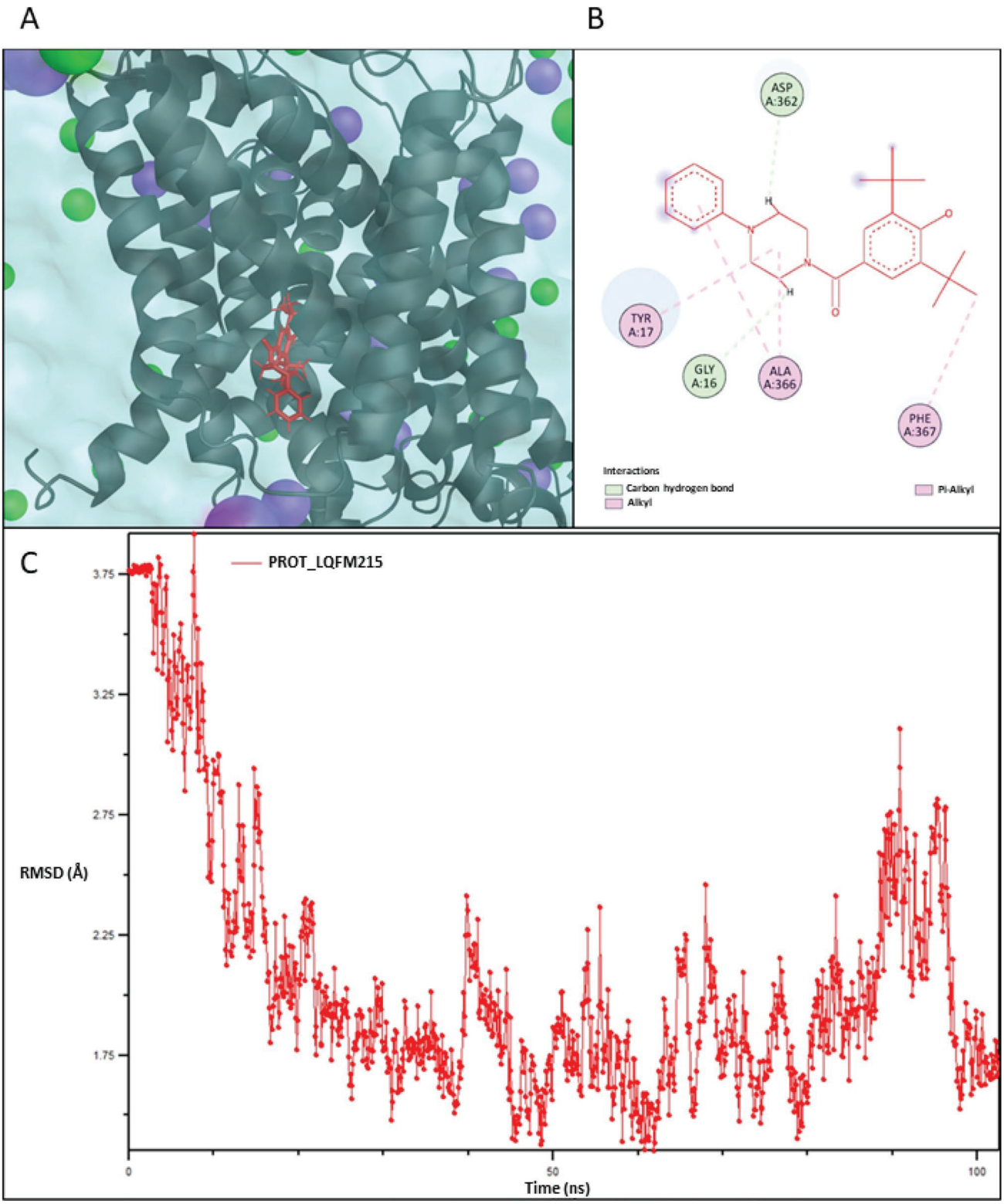

Fig. 1 Molecular dynamics of PROT and LQFM215 indicate drug-target interaction. **A** The dynamic system is composed of the PROT protein (shown in grey), the LQFM215 ligand (depicted in red), along with sodium (purple) and chloride (green) ions in the simulation water box (represented in cyan). **B** This panel depicts the interaction of LQFM215 with the amino acid residues at the active site, with emphasis on GLY16 and ASP362, which form hydrogen bonds. **C** This graph displays the RMSD values over the course of the 100 ns molecular dynamics simulation. Each point on the graph corresponds to an RMSD value at a particular frame in the simulation

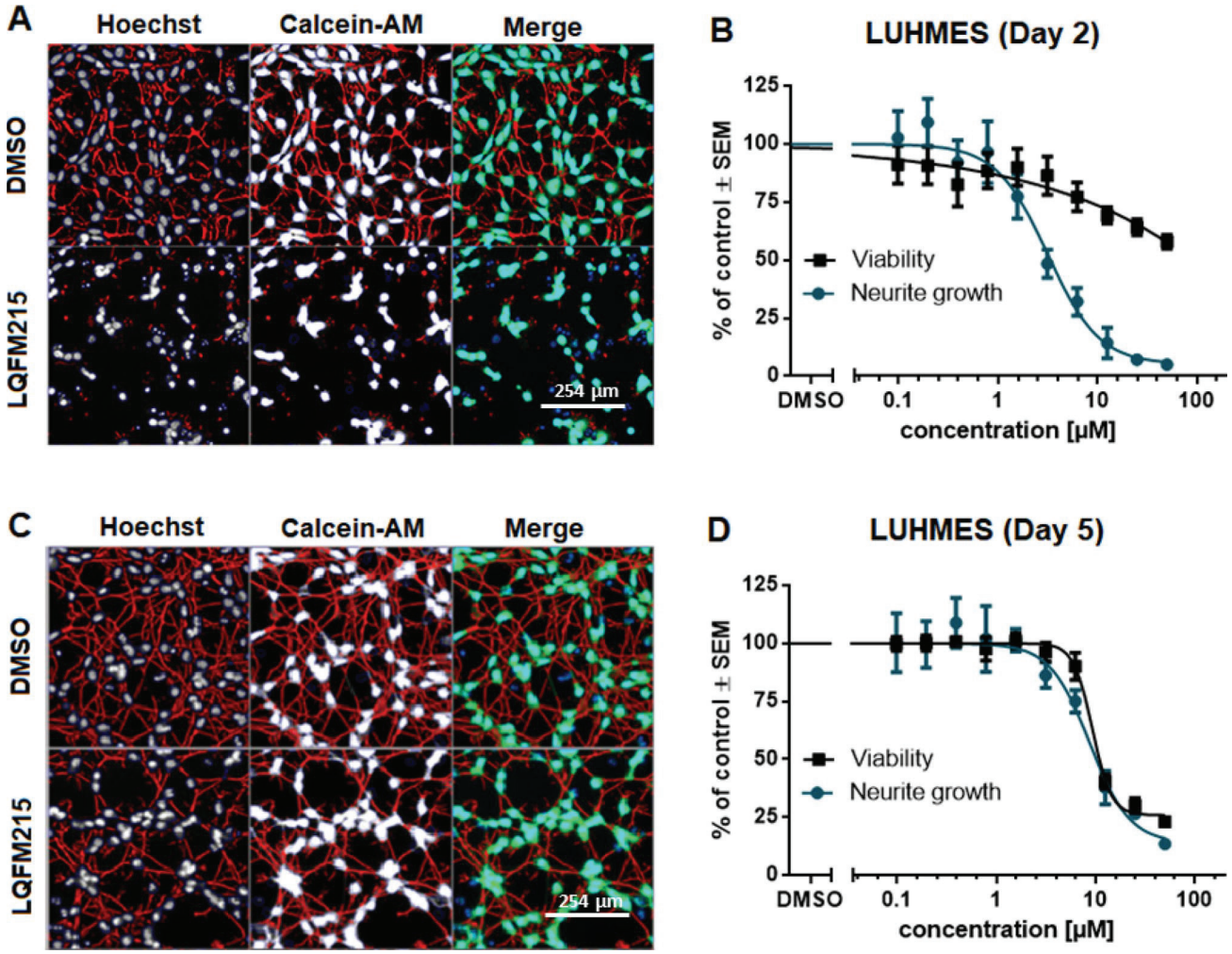

Fig. 2 Proline transporter inhibitors impact neurite development in LUHMES Cells. Images were acquired after staining with Hoechst H-33,342 (blue) and calcein-AM (green) to calculate neurite area and viability. Double positive cells (blue and green) were counted as viable. Figure shows representative images of the impact of compounds on neurite outgrowth (red), neurite integrity and viability of LUHMES cells, when applied at approximately concentration of their respective EC_{50} values. **A**, **B** This panel provides representative images and concentration-response curves of cell viability and neurite growth in LUHMES cells with developing neurites (day 2), both untreated (DMSO) and treated with LQFM215 (6 µM). **C**, **D** Representative images and concentration-response curves depicting cell viability and neurite growth in LUHMES cells with mature neurites (day 5) are shown, both for untreated (DMSO) and treated conditions. Compounds were added across a concentration range from 100 to 0.09 µM (LQFM215) with 1:2 dilution steps in a DMSO-containing medium. The final concentration of DMSO was consistently at 0.1% and used as the solvent control. Cell viability, neurite area, and neurite growth were evaluated through high content image analysis. The data presented here is representative of three independent experiments, each conducted in triplicate

treated on day-2 with LQFM215 for 24 h at concentrations maintaining cell viability above 80% but reducing neurite growth. After the 24 h treatment, the medium was carefully replaced with fresh, warm DM+/+ media, and the cells were cultured for another 24 h. A parallel culture of pre-differentiated LUHMES cells (day-2) was maintained under the same treatment, without medium replacement. Following the medium replacement after treatment with LQFM215 and a subsequent 24 h period, recovery of the neurite area was observed (Fig. 3B), indicated by an increase in IC50 values (> 25µM). While these findings should be interpreted with caution due to the limitations of in vitro models, they provide an optimistic perspective for LQFM215, due to potential for neurite growth recovery after medium removal.

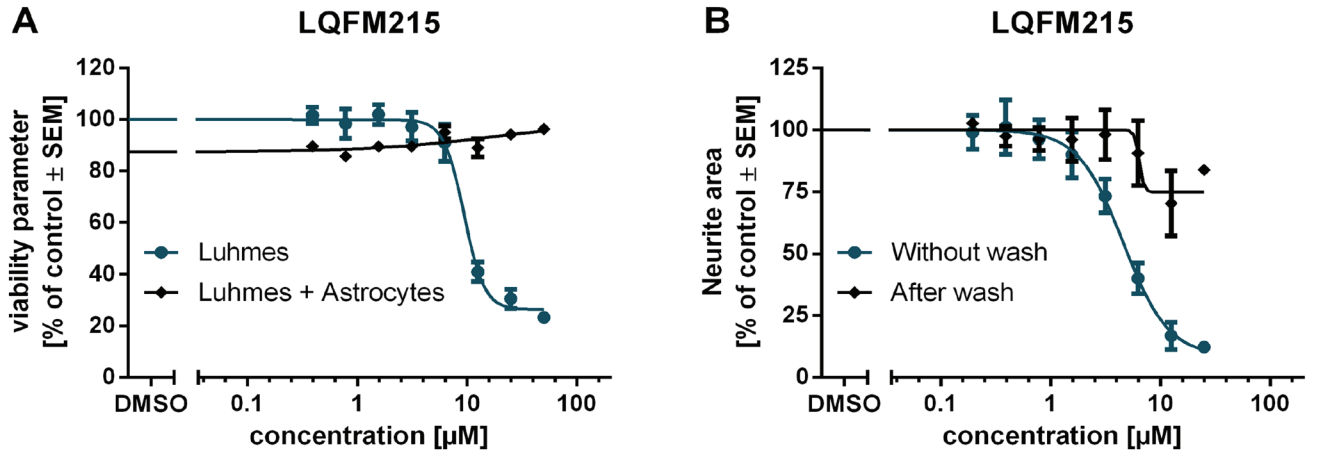

Fig. 3 Astrocytes and LUHMES cell washing prevent cytotoxicity from high LQFM215 concentrations. **A** Pre-differentiated LUHMES cells (day 2) were seeded onto astrocyte cultures at a density of 100,000 cells/cm. The cells were treated with LQFM215 for 24 h, and cell viability was assessed by measuring LDH release. Representative data from three experiments performed in triplicate are shown. Additionally, LUHMES cells cultured alone were incubated with varying concentrations of LQFM215 for 24 h, and cell viability was evaluated using calcein-AM labeling followed by high-content image analysis. Representative data from four experiments performed in triplicate are shown. **B** LUHMES cells were pre-differentiated in differentiation media containing glucose for 2 days. On day 2, the cells were seeded at a density of 45,000 cells/cm and treated with LQFM215 for 24 h at concentrations that significantly inhibited neurite area but maintained cell viability above 80%. After the 24 h treatment, the medium was carefully replaced with fresh/warm differentiation media, and the cells were cultured for an additional 24 h. In parallel, as a control, LUHMES cells were cultured without replacing the medium for 24 h. Neurite growth was evaluated using high-content image analysis. Representative data from three experiments performed in triplicate are shown

Proline Transporter Inhibitor Presents Antipsychotic Effect

Motor Assessment in the Open Field test

Given that LQFM215 has been identified as a PROT inhibitor and a neuroactive compound, we assessed its effect in a schizophrenia model characterized by a glutamatergic deficit. We examined the influence of LQFM215 on psychotic-like behaviors induced by ketamine in the Open field test (Fig. 4). Ketamine treatment (50 mg/kg) induced hyperlocomotion in mice, as indicated by an increased number of crossings and center entries compared to control animals. LQFM215 (20 and 30 mg/kg) reduced the number of crossings (13.56 ± 2.683 and 16.11 ± 1.252, One-way ANOVA); $F(4, 40) = 9.656$, $p = 0.0025$ and increased immobile time at the same doses compared to ketamine-treated control mice (128.7 ± 28.84 and 193.6 ± 21.30, One-way ANOVA); $F(4, 41) = 13.52$, $p = 0.00010$ (Fig. 4A and C). LQFM215 treatment decreased the number of center entries at all doses (14.74 ± 3.283; 8.988 ± 2.622 and 13.20 ± 1.862, One-way ANOVA); $F(4, 37) = 5.586$, $p = 0.0013$ compared to the ketamine-treated mice (Fig. 4B). In terms of grooming behavior, LQFM215 led to an increase at the highest dose (5.375 ± 0.5324, One-way ANOVA); $F(4, 38) = 5.607$, $p = 0.0012$ (Fig. 4D). These results suggest that LQFM215 may counteract ketamine-induced hyperlocomotion in mice, thereby demonstrating potential antipsychotic properties.

Assessment of Social Interaction in the Three-Chamber Test

The ketamine-induced psychosis model also impacts social behavior in mice. As observed in the three-chamber test, animals treated with ketamine spent more time in the empty chamber and less time in the chamber with the intruder animal (Fig. 5A, B). Despite a clear trend towards aligning with the behavior of the saline control group, LQFM215 did not significantly alter the time spent in different chambers across all tested doses. However, when considering the time spent in social interactions, mice subjected to ketamine-induced psychosis and treated with 10, 20, and 30 mg/kg LQFM215 spent more time engaging with the intruder animal compared to the positive control group treated only with ketamine (103.9 ± 9.936; 125.5 ± 17.92 and 106.8 ± 19.89, One-way ANOVA); $F(4, 43) = 5.427$, $p = 0.0013$ (Fig. 5D). Notably, at the dose of 30 mg/kg, LQFM215 also significantly increased the number of social interactions (touches and/or sniffs to the pencil cup with a mouse) in this model (19.67 ± 2.319, One-way ANOVA); $F(4, 33) = 3.319$, $p = 0.021$ (Fig. 5C). Collectively, these results suggest that LQFM215 has potential in alleviating negative symptoms in a ketamine-induced model of psychosis.

Assessment of PPI

PPI is deemed a gold standard for animal models of schizophrenia, primarily because it mimics a deficit in the ability to

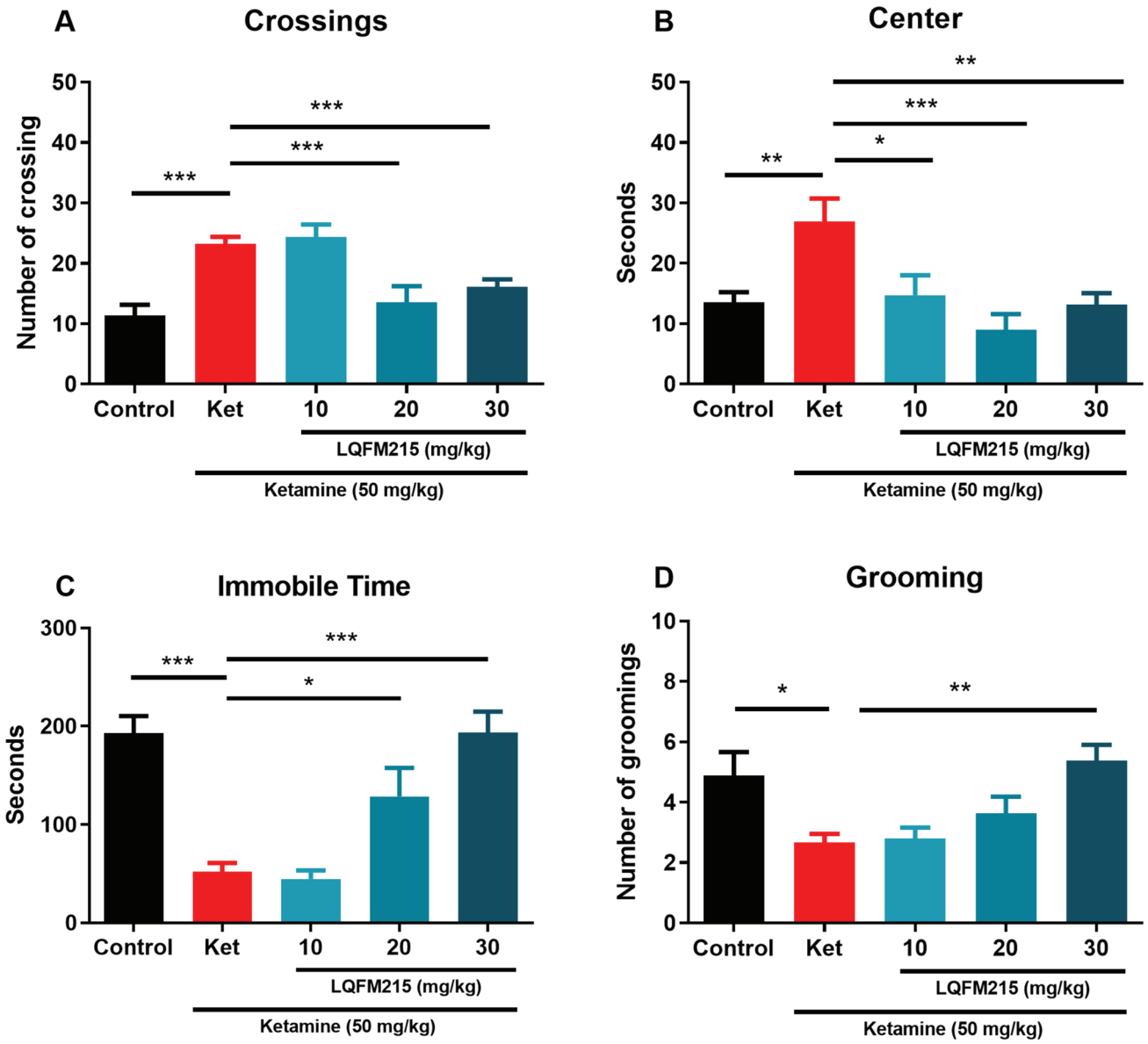

Fig. 4 LQFM215 reduces hyperlocomotion in the open field in a ketamine-induced psychosis model. This figure presents graphs representing exploration parameters in the open field test following the induction of ketamine-induced psychosis. **A** Number of crossings in the open field apparatus. **B** Time spent in the center of the apparatus. **C** Time spent immobile in the apparatus. **D** Number of grooming behaviors observed after 10 min. All data are presented as mean ± SEM. Statistical significance is indicated as *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, compared to the positive control group. The data were analyzed using the one-way ANOVA test followed by Dunnett's post-test. Each group consisted of 10 animals

inhibit responses to irrelevant or unimportant stimuli, known as a sensory gating deficit. This deficiency in response inhibition is perceived as one of the core symptoms of schizophrenia and may correlate with the cognitive symptoms of the disorder. For our model, we induced schizophrenia-like symptoms by administering ketamine (50 mg/kg) and then treated the animals with LQFM215. We observed that treatment with the transporter inhibitor at all doses increased PPI indices (54.09 ± 6.187; 65.45 ± 5.154 and 54.13 ± 5.339, One-way ANOVA); F (4, 63) = 7.061, p = 0.00014 (Fig. 6). Collectively, these findings suggest that LQFM215 may ameliorate the cognitive symptoms induced by ketamine.

Discussion

This study provides novel insights into the behavior of the PROT-LQFM215 complex in molecular dynamics simulations, as well as the impacts of LQFM215 on dopaminergic neurons and schizophrenia models. In our previous study, we

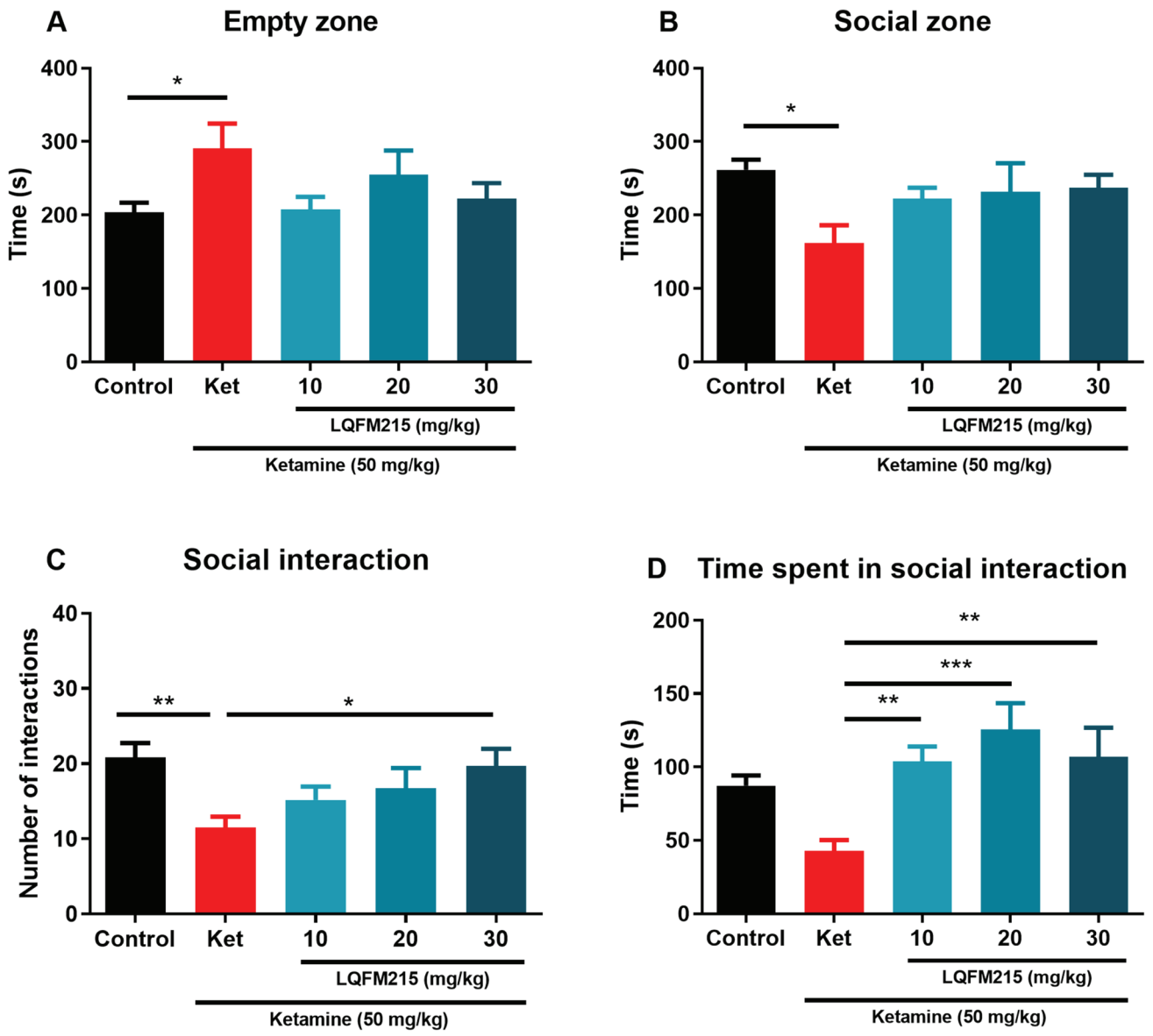

Fig. 5 LQFM215 improves social interactions in a ketamine-induced psychosis model. This figure presents graphs representing social interaction and apparatus exploration in the three chambers test following the induction of ketamine-induced psychosis. **A** Time spent in the empty zone (center) of the three chambers apparatus. **B** Time spent in the zone with a new animal in the three chambers apparatus. **C** Number of interactions between the experimental animal and the intruder animal (touches and/or sniffs to the pencil cup). **D** Time spent in social interactions between animals (touches and/or sniffs to the pencil cup). All data are presented as mean ± SEM. Statistical significance is indicated as *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, compared to the positive control group. The data were analyzed using the one-way ANOVA test followed by Dunnett's post-test. Each group consisted of 10 animals

built a model of the PROT protein (SLC6a7) and performed molecular docking with the compound LQFM215, which showed an adequate fit in the transporter binding site [15] and a better score when compared to specific inhibitors such as those cited in Zipp's (2014) and Yu and collaborators [31, 32]. Here, our molecular dynamics analysis revealed that the PROT-LQFM215 complex exhibits an average RMSD of 2.069 Å ± 0.549 over a 100ns molecular dynamics simulation, with peak deviation occurring in the early stages, likely corresponding to the system's equilibration phase. This suggests the protein-ligand complex formed during docking maintains its structural integrity over the simulation period. Thus, our molecular dynamics simulations complement the docking analysis, demonstrating that LQFM215 remains stably bound to the PROT protein, substantiating the potential of LQFM215 as a PROT inhibitor.

The concentration of proline in the intracellular or extracellular space may display different effects on neuronal

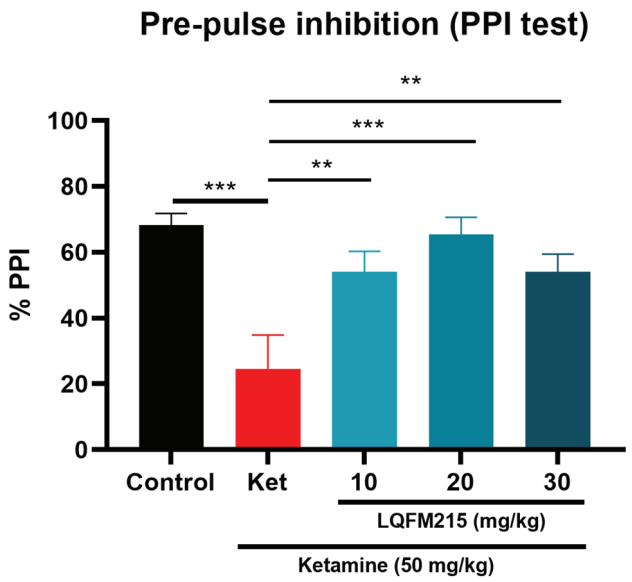

Fig. 6 LQFM215 improves pre-pulse inhibition test response in a ketamine-induced psychosis model. This figure presents a graph representing pre-pulse stimulus inhibition in the PPI test following the induction of ketamine-induced psychosis and treatment with LQFM215. All data are presented as mean ± SEM. Statistical significance is indicated as **$p < 0.01$ and ***$p < 0.001$, compared to the positive control group. The data were analyzed using the one-way ANOVA test followed by Dunnett's post-test. Each group consisted of 10–15 animals

function. Unfortunately, only a few pharmacological tools are available for studying this neurochemical dynamic. The mitochondrial abnormality in the rodent model of proline dehydrogenase 1 (PRODH), leads to an increase in L-proline concentration and can induce cognitive deficits and startle response alterations in mice during the neurodevelopment [33, 34]. In rodents, the pharmacological model of hyperprolinemia, which is induced in young rodents and analyzed in adult animals, alters glutamatergic homeostasis in the brain and is associated to oxidative stress and cognitive alterations [35, 36]. This data is consistent with those observed in humans, where the genetic condition of hyperprolinemia is associated with schizoaffective disorders and schizophrenia [37].

In contrast with these evidence [13], knockout mouse for PROT (SLC6A7) do not present cognitive deficits, or disruption in PPI, or altered L-glutamate uptake, or neurotoxicity [10, 13]. Despite multiple studies demonstrating that the hyperprolinemia model illustrates deleterious effects of high L-proline doses on the central nervous system, the precise deletion of PROT (SCL6A7) does not yield disruptive effects on animal behavior or neural tissue. This gap in our understanding of PROT's function, coupled with the consequences of its specific inhibition, underscores its fundamental role in neurochemistry and its potential as a pharmacological target.

Here, we analyzed the impact of the new PROT inhibitors on the cell viability of LUHMES cell line with display characteristics of dopaminergic neurons [22]. Numerous processes that evolve into dendrites and axons are extended by neurons and neuronal-like cells during development. These "neurites" are essential for neurotransmission [22]. We found that LQFM215 exhibits cytotoxicity against the LUHMES cell line, with impact on neurite formation and cell viability. The impact was more pronounced on neurite formation, as indicated by a decrease in neurite area with LQFM215 treatment at concentrations that result in 50% inhibition (IC50:3μM). However, this cytotoxicity was mitigated in co-culture with astrocytes, suggesting that the drug might have different effects in a more complex and physiological environment, such as the brain tissue, where astrocytes are present. Furthermore, the effects on neurite growth appear to be reversible, a potentially significant finding when considering the potential side effects of LQFM215. In contrast, PROT KO mice do not present toxic extracellular concentrations of L-proline in the brain [10]. Taken together, experiments with LUHMES cells indicate that these compounds may be safe to use on in vivo approaches when neurons are surrounded by astrocytes.

In our latest study we demonstrate that the L-proline transporter inhibitor LQFM215 reduces neuronal loss and motor impairment in a mouse model of ischemic stroke [15]. This suggests that the inhibitor may protect brain cells from the programmed cell death that occurs after an ischemic stroke. In our previous work, we observed a reduction in animal locomotion, as observed in PROT KO animal [10]. However, the precise process by which this occurs is still unknown.

Based on the evidence presented that pharmacological and genetic alteration of PROT function may interfere in mice locomotion [10, 15], we decided to investigate the effect of PROT inhibition in a schizophrenia model. Here, we used the ketamine-induced psychosis model, which is characterized by mouse hyperlocomotion and reduced social interaction when compared to control animals. Ketamine is known for inducing psychosis-like symptoms through competitive antagonism of NMDAR, modeling the glutamatergic hypothesis of schizophrenia, impairing parameters of memory and social interaction as negative symptoms [1, 28].

In our model of schizophrenia, LQFM215 effectively mitigated ketamine-induced hyperlocomotion, as demonstrated in open-field testing, at doses of 20 and 30 mg, indicating its potential to alleviate positive symptoms associated with schizophrenia, including heightened agitation. This finding aligns with the study conducted by Kumar et al., which showed that risperidone, a medication used in schizophrenia treatment, also reduced locomotion in the ketamine-induced model. Hence, LQFM215 exhibited a comparable effect to risperidone [38]. Although a decrease in the time spent in the center was observed in animals treated with LQFM215

across all three doses, this does not signify an anxiety-related behavior. Previous studies have demonstrated that this parameter is associated with reduced locomotion [15]. Additionally, we observed a decrease in self-grooming due to ketamine administration. However, the 30 mg/kg dose of LQFM215 was able to reverse this effect and restore self-grooming behavior to levels similar to those seen in control animals. According to certain authors, self-grooming in animals is an inherent behavior necessary for maintaining cleanliness and facilitating various physiological functions, such as thermoregulation, social interaction, and stimulation [39–41]. This demonstrates the ability of the 30 mg/kg dose of LQFM215 to reinstate normal behavior in animals.

This potential therapeutic role of LQFM215 was further supported by its influence on social behavior in mice subjected to ketamine-induced psychosis. Mice treated with LQFM215 spent more time engaging with the intruder animal, indicating a potential amelioration of negative symptoms, another key aspect of schizophrenia. This finding aligns with the observations made by Mehrnoosh and colleagues, indicating a comparable impact on social behavior resulting from the administration of clozapine and risperidone, both being medications employed for treating schizophrenia. These drugs, along with LQFM215, demonstrated an augmentation in the duration of social interaction with unfamiliar animals [42].

Most importantly, LQFM215 administration led to increased PPI indices. This effect was observed at all doses tested, signifying a potential mitigation of the sensory gating deficit commonly seen in schizophrenia. This sensory gating deficit is considered one of the core symptoms of schizophrenia and correlates with the cognitive symptoms of the disorder. Hence, the observed improvement in PPI indicates that LQFM215 may play a role in alleviating cognitive symptoms induced by ketamine. This is significant, given that cognitive deficits in schizophrenia are often resistant to treatment and are strongly linked to functional outcomes.

The strategy of inhibiting other transporters to modulate glutamatergic neurotransmission and reverse the symptoms of schizophrenia has already been studied, such as in the case of type 1 glycine transporters (GlyT1). N[3-(4′-fluorophenyl)-3-(4′-phenylphenoxy)propyl]sarcosine (NFPS), for example, demonstrated beneficial effects in an animal model of schizophrenia by increasing PPI of the acoustic startle response in DBA/2J mice, a strain with low baseline levels of PPI [43]. Other inhibitors have shown efficacy in treating both positive and negative symptoms, normalized spontaneous PPI deficits in DBA/2 mice, and reversed amphetamine-induced locomotor hyperactivity, such as SSR504734 and methyl benzamide derivatives [44, 45], what we can observe with the LQFM215 in our work. It is important to note that the precise regulation of L-proline content in the central nervous system (CNS) and the role of the SLC6A7 transporter (PROT) have already been investigated. Studies have demonstrated an association between elevated levels of L-proline in the cerebrospinal fluid and schizophrenia [46], while transcriptomic analyses have revealed diminished expression of the PROT gene in post-mortem autistic brains [47]. These studies demonstrate that modulation of the glutamatergic pathway through these transporters can reverse symptoms of schizophrenia and serve as a target for the discovery of new drugs, where the greatest challenge currently is improving negative symptoms of the disease, making inhibitors of proline transporters and the study of their functioning interesting for new drugs development.

Regarding the limitations of this study, it is currently premature to determine the selectivity of the LQFM215 compound in relation to other proline transporters or members of the SLC6 family. Additionally, toxicological studies are still necessary to understand the consequences of PROT inhibition in peripheral organs and the animal metabolism. Lastly, long-term treatments are required to ascertain the efficacy and safety of PROT inhibition in chronic models of schizophrenia. Despite these considerations, our research unequivocally points to a novel therapeutic target with potential antipsychotic effects in acute murine models of schizophrenia.

Conclusion

In conclusion, this study undertook a comprehensive examination of the interaction between the PROT-LQFM215 complex using molecular dynamics simulations. The analysis revealed consistent structural stability, confirming the persistence and stability of the protein-ligand complex. Furthermore, our investigation into the effects of PROT inhibition on LUHMES cells indicated a reversible and concentration-dependent impact on neurite growth and cell viability. Notably, the cytotoxic effects of LQFM215 were attenuated in the presence of astrocytes, suggesting a potential protective role of these cells.

Moving beyond cellular models, our study explored the antipsychotic potential of LQFM215 in murine models of schizophrenia. Notably, LQFM215 demonstrated the ability to mitigate ketamine-induced hyperlocomotion, restore social interaction deficits, and improve pre-pulse inhibition, all of which are indicative of ameliorated psychotic-like behaviors. These findings suggest that LQFM215 holds promise as a therapeutic candidate for addressing aspects of schizophrenia, particularly in terms of its potential to alleviate cognitive and negative symptoms. However, the complexities and nuances of such effects necessitate further investigation.

Author Contributions GAC, RAC, JFCP, DSA, ALBR, OCOL, LML, EMSF, SS, have made substantial contributions to acquisition of data and data analysis. EMSF, SS, ML, EADBG, RSG, AB and MCXP have made substantial contributions to conception, design, and interpretation of data. AB, ML, RM, MCXP, were involved in drafting the manuscript or revising it critically for important intellectual content. MCXP gave the final approval of the version to be published.

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Data Availability The statement in the text is accurate and complies with the journal's requirement for data availability.

Declarations

Conflict of interest The authors declare no conflicts of interest.

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