

## RESEARCH ARTICLE



# Repurposed catechol-O-methyltransferase inhibitors tolcapone and entacapone preferentially suppress aggregation and seeding of P301 mutant tau in human neuronal models

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**Background and purpose:** Pathogenic aggregation and propagation of seed-competent TAU assemblies drive tauopathies. MAPT P301 mutations accelerate aggregation and enhance seed competence, yet pharmacological strategies selectively targeting these pathogenic species remain limited. We investigated whether the clinically approved catechol-O-methyltransferase inhibitors tolcapone (TOL) and entacapone (ENT) preferentially modulate mutant TAU aggregation and seeding.

**Experimental Approach:** TOL and ENT effects on TAU aggregation were evaluated via cell-free assays, surface plasmon resonance (SPR), and in silico docking. Functional consequences of compound-modified fibrils were assessed in mutant TAU-expressing SH-SY5Y cells. Translational relevance was examined in human induced pluripotent stem cell (hiPSC)-derived neurons exposed to pathogenic K18 fibrils, followed by post-seeding compound treatment.

**Key Results:** Both compounds dose-dependently inhibited TAU aggregation, exhibiting greater potency, stronger SPR binding affinities, and more favourable computed interaction energies for P301S mutant versus wild-type TAU. Fibrils formed with TOL or ENT induced less downstream TAU oligomerization and phosphorylation in SH-SY5Y cells, with TOL showing superior protection. In hiPSC-derived neurons,

**Abbreviations:** AUC, area under the curve; BCA, bicinchoninic acid; BDNF, brain-derived neurotrophic factor; COMT, catechol-O-methyltransferase; CTL, control; DAPI, 4',6'-diamidino-2-phenylindole; EGFP, enhanced green fluorescent protein; ENT, entacapone; hiPSC, human induced pluripotent stem cell; iN, induced neuron; LDH, lactate dehydrogenase; MAPT, microtubule-associated protein tau gene; MM-GBSA, molecular mechanics-generalized Born surface area; NGN2, neurogenin 2; NT-3, neurotrophin-3; RNP, ribonucleoprotein; RT-PCR, reverse-transcription PCR; SPR, surface plasmon resonance; TBS, Tris-buffered saline; TBST, Tris-buffered saline containing Tween-20; ThT, thioflavin T; TOL, tolcapone.

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post-seeding treatment with either compound decreased fibril-induced, sarkosyl-insoluble, TAU aggregation and phosphorylation without overt cytotoxicity.

**Conclusion and Implications:** TOL and ENT preferentially reduced the aggregation and seeding of pathogenic P301 mutant TAU in the experimental models examined. These findings provide proof-of-concept support for mutation-focused pharmacological approaches and identify catechol scaffolds as starting points for developing more potent and safer TAU-directed compounds. Further studies are required to define the relevant molecular interactions, confirm the neuronal findings across additional hiPSC lines and determine whether effective CNS exposure can be achieved in vivo.

#### KEYWORDS

catechol-O-methyltransferase inhibitors, drug repurposing, human iPSC-derived neurons, P301 mutation, TAU aggregation, tauopathy, tolcapone

## 1 | INTRODUCTION

Intracellular aggregation of the **microtubule-associated protein TAU** defines tauopathies (Strang et al., 2019). This pathology emerges early, spreading progressively through neuronal networks in a prion-like manner, driving disease progression and clinical heterogeneity (Cope et al., 2018; Franzmeier et al., 2021; Kaufman et al., 2018; Kim et al., 2020; Vogel et al., 2020). Because TAU pathology often precedes substantial neuronal loss and brain atrophy, pathogenic TAU species are considered upstream drivers of neurodegeneration rather than passive by-products (La Joie et al., 2020; Xia et al., 2017).

Specific TAU assemblies act as seeds, templating the conversion of soluble TAU into aggregation-prone conformers to enable cell-to-cell spread (Brunello et al., 2019). Unlike the low-level aggregation seen in normal ageing, tauopathies feature the accumulation of stable, seed-competent TAU species that propagate efficiently (Vogel et al., 2020). This seeding and propagation is influenced by neuronal activity, cellular stress, and MAPT gene mutations (Strang et al., 2019).

TAU aggregation is primarily driven by its microtubule-binding domain, specifically the amyloidogenic motifs VQIINK (PHF6\*) and VQIVYK (PHF6), which are central to fibril formation and seeding (Ganguly et al., 2015; Li & Lee, 2006; Stöhr et al., 2017). In wild-type (WT) TAU, local secondary structure partially shields VQIVYK, limiting spontaneous aggregation (Mirbaha et al., 2018). Disrupting this structure via post-translational modifications or mutations exposes these sequences, enhancing aggregation (Chen et al., 2019).

Mutations at residue P301 (e.g., P301L and P301S) are highly pathogenic. They destabilize the R2–R3 interface, weakening interactions that restrain  $\beta$ -sheet formation, thereby accelerating aggregation and seed formation (Chen et al., 2019; Fischer et al., 2009). P301-mutant assemblies show greater stability, seeding activity and clearance resistance than WT TAU. Accordingly, cryo-electron microscopy of tauopathy filaments reveals VQIVYK as a core structural element in pathogenic fibrils (Falcon et al., 2018; Fitzpatrick et al., 2017).

Many aggregation inhibitors (small molecules, peptides and antibodies) that succeed in vitro fail to block TAU seeding and

### What is already known?

- P301 mutations enhance TAU aggregation and seeding in tauopathies.
- Tolcapone (TOL) and entacapone (ENT) are approved COMT inhibitors with anti-aggregation potential.

### What does this study add?

- TOL and ENT preferentially inhibit P301-mutant TAU aggregation and seeding.
- Post-seeding treatment reduces insoluble TAU pathology in human induced pluripotent stem cell-derived neurons.

### What is the clinical significance?

- Catechol scaffolds may guide safer TAU-directed compounds with improved brain exposure.
- Current data do not support immediate clinical repurposing of TOL or ENT.

propagation in cellular or in vivo systems (Hill et al., 2022; Soeda et al., 2019; Soeda & Takashima, 2020). This highlights the need to specifically suppress seed-competent TAU species rather than just general aggregation. Structure-based inhibitors targeting motifs such as VQIVYK show promise for blocking seeding, even against patient-derived aggregates (Seidler et al., 2018, 2019; Zheng et al., 2011).

Catechol-containing compounds, including clinically approved **catechol-O-methyltransferase** (COMT) inhibitors like **tolcapone** (TOL)

and entacapone (ENT), interfere with TAU aggregation. Early cell-free studies showed they inhibit PHF6 hexapeptide aggregation, identifying the nitrocatechol moiety as a key pharmacophore (Mohamed et al., 2013). Later work found catechols suppress TAU oligomerization via cysteine-dependent interactions and reduce pathological accumulation in P301L TAU cell and animal models (Soeda et al., 2015). However, these studies focused on bulk aggregation or early oligomers, leaving it unclear if these compounds modulate seed-competent TAU assemblies or distinguish between WT and mutant conformers.

Here, we investigate whether TOL and ENT preferentially suppress the aggregation and seeding of pathogenic P301 TAU. Using biochemical assays, SH-SY5Y cells and human induced pluripotent stem cell (hiPSC)-derived neurons expressing P301L TAU, we assess their effects on aggregation kinetics, seed competence, and intracellular propagation. Our findings provide evidence that clinically approved COMT inhibitors preferentially modulate P301-mutant TAU assemblies and support further investigation of mutation-focused approaches targeting seed-competent TAU.

## 2 | METHODS

### 2.1 | Materials and reagents

TAU peptides corresponding to WT-R2R3 and P301S-R2R3 TAU constructs were synthesized and handled as described previously (Goud et al., 2025). TOL (Sigma-Aldrich, St. Louis, MO, USA, Cat# SML0150) and ENT (Sigma-Aldrich, Cat# SML0654) were dissolved in HPLC-grade dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Cat# D8418) and stored at  $-80^{\circ}\text{C}$ . All intermediate drug dilutions were prepared in Gibco™ 1 × Phosphate Buffered Saline (PBS), pH 7.4 (Gibco, Thermo Fisher Scientific, Waltham, MA, USA; Cat# 10010023), supplemented with 0.05% Tween-20 and 5% DMSO. Unless otherwise indicated, compounds were added in a volume-normalized manner such that the final DMSO concentration did not exceed 0.125% (v/v) in any condition, including vehicle controls.

### 2.2 | In vitro TAU aggregation assay

TAU aggregation kinetics were monitored using thioflavin T (ThT) fluorescence assays as described previously (Goud et al., 2025). TAU peptides stored at  $-80^{\circ}\text{C}$  were thawed on ice and centrifuged at 18,300 g for 45 s before use. Aggregation reactions were prepared in aggregation buffer (20-mM Tris-HCl, pH 7.4, 100-mM NaCl, 1-mM EDTA; pH 7.4) containing 15- $\mu\text{M}$  ThT (Sigma-Aldrich; Cat# T3516) and 50- $\mu\text{M}$  TAU peptide, in the absence or presence of TOL or ENT at concentrations ranging from 0.01–100  $\mu\text{M}$ . The final reaction volume was 40  $\mu\text{L}$  per well in clear-bottom, black 384-well ViewPlates (Revvity, Inc., Waltham, MA, USA; Part# 6007460). To prevent evaporation, plates were sealed with TopSealA-PLUS (Revvity, Inc.; Part# 6050185).

Plates were incubated at  $37^{\circ}\text{C}$  with constant orbital agitation (1-mm amplitude, 432 rpm) in Tecan Infinite® 200 PRO multimode plate reader (Tecan Group Ltd., Männedorf, Switzerland; RRID:SCR\_020543), and ThT fluorescence ( $\lambda_{\text{ex}}$  440 nm,  $\lambda_{\text{em}}$  485 nm, number of flashes 100) was recorded every 10 min for up to 30–35 h.

Aggregation kinetics were analysed using GraphPad Prism version 10 (GraphPad Software [Dotmatics], San Diego, CA, USA; RRID:SCR\_002798). ThT fluorescence-time profiles were used to calculate the area under the curve (AUC) for each condition as an integrated measure of aggregation. AUC values were normalized to the untreated control (CTL). Concentration–response curves were generated by plotting normalized AUC values against compound concentration and fitted using three-parameter logistic models to derive half-maximal inhibitory concentration ( $\text{IC}_{50}$ ) values.

### 2.3 | Transmission electron microscopy (TEM)

Aggregation end-point samples were analysed by TEM to assess fibril morphology. Samples of WT and P301S R2R3 TAU constructs aggregated in the absence or presence of TOL or ENT were prepared for imaging after 48 h of incubation.

Aliquots (2–3  $\mu\text{L}$ ) were applied to glow-discharged carbon-coated copper grids and incubated for 1.5 min. Excess liquid was removed with filter paper, and grids were air-dried prior to imaging.

Grids were imaged using a Tecnai G2 F20 transmission electron microscope equipped with an Eagle 4K CCD camera (FEI Company, Hillsboro, OR, USA; RRID:SCR\_021365). Approximately 15 images were acquired per sample from multiple grid regions. Fibril morphology was assessed qualitatively, and fibril parameters were analysed using ImageJ software (National Institutes of Health [NIH], Bethesda, MD, USA; RRID:SCR\_003070).

Fibril width was quantified by measuring the edge-to-edge distance perpendicular to the fibril axis using the NIH ImageJ-based line tool and macro-assisted workflow. Images with a standardized scale were analysed by manually drawing two lines, each with an identical number of points, along opposing fibril edges, oriented parallel to the fibril axis. The macro automatically generated perpendicular distance measurements between corresponding points and calculated an average fibril width per measurement. Multiple fibrils were measured per image, pooled per condition for statistical analysis and the NIH ImageJ macro used for this analysis is provided as supplementary code via Zenodo (Data Availability Statement).

### 2.4 | Surface plasmon resonance (SPR)

SPR measurements were performed essentially as described previously (Goud et al., 2025), using a Sierra SPR-24 Pro system (Bruker Daltonics, Bremen, Germany) at  $25^{\circ}\text{C}$ . Monomeric WT-R2R3 and P301S-R2R3 TAU peptides were immobilized on High-Capacity Amine Sensor Surfaces (Bruker, Part #1862614) by EDC/NHS amine

coupling. PBS (pH 7.4) containing 0.05% Tween-20 and 5% DMSO was used as the running buffer.

TOL and ENT were injected over immobilized peptides using a multi-injection cycle-kinetics format (Goud et al., 2025). Sensorgrams were corrected by reference-spot subtraction, blank-injection subtraction, and DMSO solvent correction. The concentration series was globally fitted to a 1:1 interaction model using SPR Analyzer 4 software to determine the association rate constant ( $k_{on}$ ), dissociation rate constant ( $k_{off}$ ), and equilibrium dissociation constant ( $K_D$ ). SPR plots were generated using OriginPro version 8.5 (OriginLab Corporation, Northampton, MA, USA; [RRID:SCR\\_014212](#)).

## 2.5 | Generation and processing of TAU aggregates for cell-based seeding assays

TAU aggregation reactions for cell seeding assays were performed in aggregation buffer without ThT, and as described previously (Goud et al., 2025). Briefly, aggregation reactions were allowed to proceed for 30–35 h, after which reaction products were pooled, and the TAU concentration was determined using a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific™, Waltham, MA, USA; Cat# 23250). Aggregation mixtures were then standardized to a final concentration of 100-nM TAU peptide, sonicated using a Branson Digital Sonifier 250 equipped with an inverted cup-horn attachment (Branson Ultrasonics Corporation, Danbury, CT, USA) at 30% amplitude, 15 s ON/15 s OFF cycles for 1 min, and used immediately for cell transduction experiments.

## 2.6 | Cell lines and human induced pluripotent stem cells

SH-SY5Y neuroblastoma cells, originally obtained from ATCC (Manassas, VA, USA; Cat# CRL-2266; [RRID:CVCL\\_0019](#)), were engineered to stably express a **doxycycline**-inducible AAVS1-integrated TAUP301L-2N4R-EGFP (Addgene plasmid # 132393; <http://n2t.net/addgene:132393>; [RRID:Addgene\\_132393](#)) as described previously (Devyatov et al., 2025). Stable integration was verified by **puromycin** (Sigma-Aldrich, Cat# P9620) antibiotic selection with limiting dilution cloning, followed by confirmation of doxycycline-inducible EGFP expression using fluorescence microscopy and Western blot analysis (Devyatov et al., 2025).

SH-SY5Y cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) high glucose (4.5 g/L) with L-glutamine (Lonza, Basel, Switzerland; Cat# 12-604F), supplemented with 10% fetal calf serum (Gibco; Cat# A5256701) and 1% penicillin-streptomycin (Thermo Fisher Scientific; Cat# 15140163), in a humidified incubator at 37°C with 5% CO<sub>2</sub>/atmospheric air. Cells were maintained at approximately 80% confluency and passaged every 3–4 days and used between Passages 3 and 15 for all experiments after thawing. Cell lines were authenticated periodically by short tandem repeat profiling (Stránská, 2022) and tested for mycoplasma contamination bi-

weekly according to established laboratory protocols (Buriánová & Kotulová, 2022).

The hiPSC line KOLF2.1 J was obtained from The Jackson Laboratory (Bar Harbour, ME, USA; Cat# JIPSC1000; [RRID:CVCL\\_B5P3](#)). hiPSCs were maintained in StemFlex medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA; Cat# A3349401) on culture dishes pre-coated with 5 µg/ml recombinant human **vitronectin** (Gibco, Thermo Fisher Scientific; Cat# A400457). Cells were passaged every 4–5 days using 0.5-mM EDTA and reseeded at a ratio of 1:5–1:10. Culture medium was refreshed according to the manufacturer's instructions. hiPSCs were routinely monitored for morphology and growth characteristics and confirmed to be mycoplasma-negative using Myco-Detect Fast qPCR Kits (Topgen Biotechnology Co., Ltd., Kaohsiung, Taiwan; Cat# QM050) prior to downstream differentiation experiments; cells were used between passages 5–15 after thawing.

## 2.7 | SY5Y-TAUP301L cell differentiation and seeding

For neuronal differentiation, SY5Y-TAUP301L cells were transferred to Neurobasal™-A medium (Gibco; Cat# A1371201) supplemented with L-glutamine (Lonza, Cat# 12-604F), 1 × Gibco™ B-27™ Supplement (50X) serum-free (Thermo Fisher Scientific; Cat# 17504044), 1% penicillin-streptomycin (Thermo Fisher Scientific), **retinoic acid** (Sigma-Aldrich Cat# R2625), and 50-ng·ml<sup>-1</sup> recombinant human **BDNF** protein (R&D Systems, Inc.; Cat# 248-BDB-250), as described previously (Devyatov et al., 2025). Expression of TAUP301L-EGFP was induced by adding 0.5-µg·ml<sup>-1</sup> doxycycline (Sigma-Aldrich, Cat# D3447) 3 days before seeding and maintained throughout the experiment.

For seeding assays, differentiated SY5Y-TAUP301L cells were transduced with TAU aggregation products at a final concentration of 100 nM using TurboFect™ transfection reagent (Thermo Fisher Scientific; Cat# R0533) in Opti-MEM™ (Thermo Fisher Scientific; Cat# 11058021), as described previously (Devyatov et al., 2025; Goud et al., 2025). Transfection mixtures were incubated for 15 min at room temperature prior to addition to cells. Cells were incubated for 72 h after transduction, then processed for Triton X-100 TAU fractionation and Western blot analysis as described below.

## 2.8 | Generation of isogenic iPSC lines using CRISPR/Cas9

Guide RNA (gRNA) sequences targeting MAPT exon 10 were designed using the web-based TrueGuide™ CRISPR gRNA Design Tool (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA). The selected gRNA (CRISPR1107020\_SGM; sequence: AATATCAAA-CACGTCCCGGG) was synthesized and assembled with Cas9 nuclease (Invitrogen) to form Cas9 ribonucleoprotein complexes (Cas9 RNPs). Cas9 RNPs were electroporated into hiPSCs using the Invitrogen™ Neon™ Transfection System (Thermo Fisher Scientific, Baltimore, MD,

USA, Cat# MPK5000). For P301L editing by homology-directed repair, Cas9 RNPs were co-electroporated with a piggyBac donor plasmid carrying the P301L mutation using the following Neon settings: 1400 V, 10 ms, three pulses (programme #22). Cells were treated with puromycin ( $1 \text{ mg} \cdot \text{L}^{-1}$ ) for 48 h starting on Day 3 post-electroporation, followed by a second 48-h puromycin selection on Day 10. Individual surviving colonies were manually picked 2 weeks after transfection, expanded and screened by PCR. Correctly edited clones were verified by Sanger sequencing by the [National Yang Ming Chiao Tung University \(NYCU\) Sequencing Services](#) (Figure S12a).

The homozygous MAPT(P301L/P301L) clone, hereafter referred to as the MAPT(P301L) clone, used for downstream neuronal experiments, was further validated for pluripotency marker expression and morphology, three-germ-layer differentiation potential by RT-PCR using the primers listed in Table S8, and NGN2-induced neuronal differentiation capacity (Figure S12B-D). Potential off-target sites for the MAPT exon 10 gRNA were predicted using ThermoFisher Invitrogen TrueDesign Genome Editor, and the top three predicted off-target sites were PCR-amplified from genomic DNA of parental KOLF2.1J hiPSCs and the MAPT(P301L) clone, followed by Sanger sequencing. Primer sequences are listed in Table S9. No detectable sequence alterations were observed in the MAPT(P301L) clone compared with the parental line at these predicted off-target sites (Figure S12e,f). For downstream neuronal experiments in this study, one validated MAPT(P301L) isogenic clone was used, and all treatment comparisons between vehicle and TOL or ENT were performed within this same genetic background. No independently edited second clone or patient-derived MAPT(P301L) line was included.

## 2.9 | Generation of induced neurons (iNs) from hiPSCs

hiPSCs were transfected with PB-TO-hNGN2 (Addgene plasmid #172115; a gift from iNDI & Michael Ward; [RRID:Addgene\\_172115](#)) and pRP[Exp]-EGFP-EF1A > hyPBse (VectorBuilder, Chicago, IL, USA; Cat# VB900088-2871jha). The transfected cells were subsequently cultured in StemFlex medium containing puromycin ( $1 \text{ mg} \cdot \text{L}^{-1}$ ) for 1 week.

For differentiation, hiPSCs were dissociated with 0.5 mM EDTA and replated onto vitronectin-coated dishes in Essential 8 medium (Gibco, Cat# A1517001) supplemented with Y-27632 ( $5 \mu\text{M}$ ;  $\text{day}^{-1}$ ). On Day 0, the medium was replaced with DMEM/F12 (Gibco, Cat# A4192002) supplemented with  $1 \times \text{N2}$  (Gibco, Cat# 17502001), MEM Non-Essential Amino Acids (Gibco, Cat# 11140050), BDNF ( $10 \mu\text{g} \cdot \text{L}^{-1}$ ; ACROBiosystems, Newark, DE, USA; Cat# BDF-H5219), neurotrophic factor NT-3 ( $10 \mu\text{g} \cdot \text{L}^{-1}$ ; ACROBiosystems, Cat# NT3-H5213), mouse laminin ( $0.1 \text{ mg} \cdot \text{L}^{-1}$ ; Gibco, Cat# 23017015) and doxycycline ( $2 \text{ mg} \cdot \text{L}^{-1}$ ). On Day 2, cells were dissociated and replated onto poly-D-lysine (Sigma-Aldrich, St. Louis, MO, USA; Cat# P0899) and laminin-coated plates at  $6\text{--}8 \times 10^4 \text{ cells cm}^{-2}$  in Neurobasal medium (Gibco, Cat# 21103049) containing  $1 \times \text{B27}$  (Gibco, Cat# 17504044), GlutaMAX (Gibco, Cat# 35050061), mouse laminin, BDNF and NT-3. [Cytarabine](#) ( $2.5 \mu\text{M}$ ; Sigma, Cat# C1768) was added on Day 5 for 48 h

to inhibit the proliferation of undifferentiated cells. Afterward, half of the medium was replaced every 3 days with fresh Neurobasal medium supplemented with B27/GlutaMAX/laminin/BDNF/NT-3.

## 2.10 | Preparation and transfection of sK18f

Pre-formed P301L mutant human TAU (K18) fibrils were purchased from GeneTex (Irvine, CA, USA; Cat# GTX17675-pro). Fibrils were diluted to  $0.2 \mu\text{g} \cdot \mu\text{L}^{-1}$  in PBS (137-mM NaCl, 3-mM KCl, 7-mM  $\text{Na}_2\text{HPO}_4$ , 1.5-mM  $\text{KH}_2\text{PO}_4$ , pH 7.4) and sonicated on ice for 10 cycles of 30-s pulses with 30-s rest at 30%–40% amplitude. Sonicated K18 fibrils (sK18f) were aliquoted and stored at  $-80^\circ\text{C}$ .

Neuronal transfection was performed using Xfect™ Transfection Reagent (Takara Bio, Kusatsu, Shiga, Japan; Cat# 631318) according to the manufacturer's instructions. For 24-well plates, each well received  $0.3\text{--}\mu\text{g}$  sK18f and  $1.5\text{--}\mu\text{L}$  Xfect; for 35-mm dishes,  $1.5\text{--}\mu\text{g}$  sK18f and  $7.5\text{--}\mu\text{L}$  Xfect were applied.

## 2.11 | Compound treatment and endoplasmic reticulum (ER) stress induction

One day after sK18f exposure, the entire culture medium was removed and replaced with Neurobasal medium supplemented with B27, GlutaMAX, mouse laminin, BDNF and NT-3, containing TOL or ENT ( $2, 10$  or  $50 \mu\text{M}$ ). Media and compounds were refreshed every 3 days and maintained for 3 weeks. To model a stress-sensitized neuronal environment relevant to tauopathy, endoplasmic reticulum stress was induced by applying  $0.4\text{--}\mu\text{M}$  [thapsigargin](#) (Sigma, Cat# T9033) for 1 week during Week 6 of differentiation (Devina et al., 2022).

## 2.12 | Fluorescent labelling of sK18f

For fluorescent labelling,  $100\text{--}\mu\text{g}$  sK18f was buffer-exchanged into  $0.1 \text{ M}$  [sodium bicarbonate](#) (pH 8.3) using an Amicon Ultra-0.5 centrifugal filter (Merck Millipore, Cat# UFC501024) and concentrated to  $45 \mu\text{L}$ . Alexa Fluor™ 647 NHS Ester (Succinimidyl Ester) ( $100 \mu\text{g}$  in  $10\text{--}\mu\text{L}$  DMSO; Invitrogen, Cat# A37573) was added ( $5 \mu\text{L}$ ) and incubated at  $25^\circ\text{C}$  with shaking at  $1500 \text{ rpm}$  on a ThermoMixer C (Eppendorf, Hamburg, Germany; [RRID:SCR\\_025397](#)). After labelling, the reaction was washed four times with  $500\text{--}\mu\text{L}$   $1 \times \text{PBS}$  using an Amicon Ultra-0.5 filter to remove unreacted dye.

## 2.13 | TAU fractionation

### 2.13.1 | Triton X-100 fractionation of SY5Y-TAUP301L cells

Triton X-100-soluble and X-100-insoluble fractions from SY5Y-TAUP301L cells were prepared as described previously (Devyatov

et al., 2025), with minor modifications. Briefly, cells were lysed in  $1 \times$  Tris-buffered saline (TBS) containing 0.05% Triton X-100, supplemented with protease and phosphatase inhibitors. Lysates were first centrifuged at  $1000 \times g$  for 10 min at  $4^{\circ}\text{C}$  to remove cell debris. The resulting supernatant was then ultracentrifuged at  $24,400 \times g$  for 1 h at  $4^{\circ}\text{C}$ . The supernatant from this step was collected and stored as the Triton X-100-soluble fraction. The remaining pellet was washed once with  $1 \times$  TBS containing 0.05% Triton X-100, supplemented with protease (Cat# 04693116001) and phosphatase (Cat# 04906837001) inhibitors from Roche (Basel, Switzerland), by centrifugation at  $24,400 \times g$  for 3 min at  $4^{\circ}\text{C}$ . The supernatant was discarded, and the final pellet was resuspended in radioimmunoprecipitation assay buffer (RIPA) buffer (supplemented with protease and phosphatase inhibitors) at the same volume as the corresponding soluble fraction and used as the Triton X-100-insoluble fraction. Protein concentration was determined in the soluble fraction using the BCA Protein Assay Kit.

### 2.13.2 | Sarkosyl fractionation of hiPSC-derived neurons

After treatment with sK18f with or without TOL or ENT, hiPSC-derived neurons were lysed in high-salt lysis buffer containing 10-mM HEPES (pH 7.5), 800-mM NaCl, 1-mM EGTA, 10% sucrose, protease inhibitor cocktail (Roche, Cat# 4693116001), phosphatase inhibitor cocktail I (TargetMol Chemicals Inc., Boston, MA, USA, Cat# C0002) and phosphatase inhibitor cocktail II (TargetMol, Cat# C0003). Cells were homogenized by pipetting, and 1/30 of the volume was added to 30% sarkosyl (Sigma, Cat# 61747) to reach a final concentration of 1%. Lysates were incubated at room temperature with gentle inversion for 1 h and centrifuged at 15,000 rpm for 1 h at  $4^{\circ}\text{C}$ . The supernatant was collected as the sarkosyl-soluble fraction. The pellet was washed once with PBS and resuspended in pellet buffer containing 10-mM HEPES (pH 7.5), 2-mM  $\text{MgCl}_2$ , 1% Triton X-100,  $1\text{-U}\cdot\mu\text{l}^{-1}$  benzonase (Merck Millipore, Darmstadt, Germany, Cat# E1014), protease inhibitor cocktail and phosphatase inhibitor cocktail I/II. The pellet was disrupted by pipetting, incubated at  $37^{\circ}\text{C}$  for 30 min to digest genomic DNA and RNA and then stored at  $-20^{\circ}\text{C}$  as the sarkosyl-insoluble fraction.

### 2.14 | Western blotting

For hiPSC-derived neurons, sarkosyl-soluble and sarkosyl-insoluble fractions prepared as described above were used directly for immunoblotting. For SY5Y-TAUP301L cells, Triton X-100-soluble and X-100-insoluble fractions were prepared as described above, and the Triton X-100-insoluble fraction was used for the detection of aggregated and phosphorylated TAU species, while the corresponding soluble fraction was used for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) detection as a loading control.

Protein samples were mixed with  $4 \times$  sodium dodecyl sulfate (SDS) sample buffer (250 mM Tris-HCl pH 8.5, 8% SDS, 40% glycerol, 2-mM EDTA, 0.075% Serva Blue G250, 0.025% phenol red and 10% 2-mercaptoethanol) and denatured at  $75^{\circ}\text{C}$  for 15 min. Denatured samples were separated on 8% SDS-PAGE gels at 70 V for 20 min, followed by 120 V for 1 h and transferred to  $0.45\text{-}\mu\text{m}$  PVDF membranes (Merck Millipore, Cat# 3010040001) in transfer buffer (25-mM Tris, 192-mM glycine, 20% methanol) at 70 V for 2.5 h.

Membranes were blocked in  $1 \times$  TBS containing 0.05% Tween-20 (TBST) and either 5% skimmed milk or 5% bovine serum albumin for 30–60 min at room temperature and incubated with appropriate primary antibodies described below overnight at  $4^{\circ}\text{C}$ . After three 10-min washes in  $1 \times$  TBST, membranes were incubated with horseradish peroxidase (HRP)-conjugated or Alexa Fluor 488-conjugated secondary antibodies for 2–4 h at  $4^{\circ}\text{C}$ , washed three times.

For reprobing, membranes were stripped with stripping buffer (1.5% glycine, pH 2.2, 1% Triton X-100, 0.1% SDS) or quenched with 3%  $\text{H}_2\text{O}_2$  in  $1 \times$  TBST for 30 min to remove residual antibodies or HRP activity, respectively, followed by two washes with TBST before the next antibody incubation.

For HRP-based detection, membranes were developed with Immobilon™ Western Chemiluminescent HRP Substrate (Merck Millipore, Cat# WBKLS0050), and chemiluminescent signals were detected with an ImageQuant LAS 4000 imaging system (GE Healthcare, Chicago, IL, USA). For fluorescence-based detection, Alexa Fluor 488 signals were detected using a ChemiDoc MP imaging system (Bio-Rad, Hercules, CA, USA, [RRID:SCR\\_019037](#)). All densitometric analyses were performed using NIH ImageJ software.

### 2.15 | Immunofluorescence staining

Cells were fixed with 4% paraformaldehyde in  $1 \times$  PBS for 20 min at room temperature, rinsed three times with PBS and permeabilized with 0.1% Triton X-100 in PBS for 5 min. Samples were blocked in  $1 \times$  PBS containing 3% bovine serum albumin (Sigma, Cat# A9418) and 1% Gibco™ Normal Goat Serum (Thermo Fisher Scientific, Cat# PCN5000) for 1 h at room temperature, followed by incubation with appropriate primary antibodies described below overnight at  $4^{\circ}\text{C}$ . After three washes with  $1 \times$  PBS, appropriate secondary antibodies were applied for 1 h at room temperature.

ProteoStat staining was then performed using the PROTEOSTAT® Aggresome Detection Kit (Enzo Life Sciences, Farmingdale, NY, USA, Cat# ENZ-51035) according to the manufacturer's instructions. After final washes, samples were mounted using VECTASHIELD® Antifade Mounting Medium (Vector Laboratories, Newark, CA, USA, Cat# H-1000; [RRID:AB\\_2336789](#)). Images were acquired using either a Zeiss LSM900 confocal microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) equipped with Airyscan 2 and an AxioCam 702 MONO camera, and a Plan-Apochromat 20x/0.8 M27 objective lens (Carl Zeiss Microscopy GmbH, Jena, Germany), or a Zeiss fluorescence microscope (Carl Zeiss Microscopy GmbH) equipped

with an Andor Zyla CMOS camera (Andor Technology, Belfast, Northern Ireland, UK; [RRID:SCR\\_023610](#)). Images were subsequently processed and analysed using NIH ImageJ software.

## 2.16 | Antibodies

Primary antibodies used for Western blotting were mouse anti-TAU-5 at 1:500 or 1:1000 (Thermo Fisher Scientific, Cat. No. AHB0042; [RRID:AB\\_2536235](#)), rabbit anti-pTAU (Ser262) at 1:1000 (Thermo Fisher Scientific, Cat. No. OPA1-03142; [RRID:AB\\_325643](#)), rabbit anti-oligomeric TAU (T22) at 1:1000 (Merck Millipore, Cat. No. ABN454; [RRID:AB\\_2888681](#)), rabbit anti-GAPDH at 1:4000 (Cell Signaling Technology, Cat. No. 2118S; [RRID:AB\\_561053](#)), mouse anti-pTAU (S202/T205; AT8) at 1:500 (Addgene, Cat. No. 216150; [RRID:AB\\_3094509](#)), rabbit anti-pTAU (S262) at 1:500 (Santa Cruz Biotechnology, Cat. No. sc-32,828; [RRID:AB\\_1129983](#)), rabbit anti-TAU at 1:4000 (GeneTex, Cat. No. GTX639564; [RRID:AB\\_3738550](#)) and mouse anti-GAPDH at 1:10,000 (GeneTex, Cat. No. GTX627408; [RRID:AB\\_11174761](#)).

Primary antibodies used for immunofluorescence were mouse anti-TAU-5 at 1:500 (Thermo Fisher Scientific, Cat. No. AHB0042; [RRID:AB\\_2536235](#)), mouse anti-pTAU (S202/T205; AT8) at 1:200 (Addgene, Cat. No. 216150; [RRID:AB\\_3094509](#)), mouse anti-OCT4 at 1:1000 (GeneTex, Cat. No. GTX627419; [RRID:AB\\_11176459](#)), rabbit anti-TUJ1 at 1:1000 (GeneTex, Cat. No. GTX637307; [RRID:AB\\_3738549](#)) and rabbit anti-NANOG at 1:1000 (Cell Signaling Technology, Cat. No. 4903; [RRID:AB\\_10559205](#)).

Secondary antibodies used for HRP-based immunoblotting were goat anti-mouse IgG-HRP at 1:10,000 (Bethyl Laboratories, Cat. No. A90-216P; [RRID:AB\\_67184](#)) and goat anti-rabbit IgG-HRP at 1:10,000 (Bethyl Laboratories, Cat. No. A120-201P; [RRID:AB\\_10631424](#)). F (ab')<sub>2</sub>-goat anti-rabbit IgG (H + L) cross-adsorbed Alexa Fluor 488 at 1:2000 (Thermo Fisher Scientific, Cat. No. A-11070; [RRID:AB\\_2534114](#)) and donkey anti-mouse IgG (H + L) highly cross-adsorbed Alexa Fluor 488 at 1:2000 (Thermo Fisher Scientific, Cat. No. A-21202; [RRID:AB\\_141607](#)) were used for fluorescence-based immunoblotting and immunofluorescence. Additional secondary antibodies used for immunofluorescence were F (ab')<sub>2</sub>-goat anti-rabbit IgG (H + L) cross-adsorbed Alexa Fluor Plus 647 at 1:500 (Thermo Fisher Scientific, Cat. No. A48285; [RRID:AB\\_2896349](#)) and goat anti-mouse IgG (H + L) highly cross-adsorbed Alexa Fluor 488 at 1:400 (Thermo Fisher Scientific, Cat. No. A-11029; [RRID:AB\\_2534088](#)). The Immuno-related procedures used comply with *British Journal of Pharmacology* recommendations.

## 2.17 | Lactate dehydrogenase (LDH) release assay

LDH release was quantified using the CytoTox 96<sup>®</sup> Non-Radioactive Cytotoxicity Assay Kit (Promega, Madison, WI, USA, Cat# G1780)

according to the manufacturer's instructions. After 4 weeks of differentiation, iPSC-derived neurons were treated with or without sk18f for 1 h, followed by administration of TOL and ENT. DMSO (0.25%, v/v) was used as the vehicle control. Neurons were subsequently maintained in culture with compounds for 5 days. To generate the 100% LDH release control, Triton X-100 was added to untreated neurons at a final concentration of 0.8% (v/v) and incubated at 37°C for 45 min. Culture supernatants were then collected and transferred to clear, flat-bottom 96-well plates. LDH assay reagent was added according to the manufacturer's protocol, and the mixture was incubated for 30 min at room temperature. The reaction was terminated by adding the stop solution, and absorbance was measured at 490 nm using the Tecan Infinite 200 Pro microplate reader.

The percentage of cell death was calculated using the following formula:

$$\text{cell death(\%)} = \frac{(\text{sample LDH release} - \text{blank}) / (\text{maximum LDH release} - \text{blank})}{\times 100}$$

## 2.18 | Live/dead cell viability assay

Cell viability was assessed based on intracellular esterase activity and plasma membrane integrity using the LIVE/DEAD<sup>®</sup> Viability/Cytotoxicity Kit for mammalian cells (Invitrogen, Cat# L3224), following the manufacturer's instructions. After 4 weeks of differentiation and maturation, iPSC-derived neurons were treated with sk18f for 1 hour, followed by incubation with TOL, ENT or DMSO vehicle control for 5 days. Cells were then stained with 0.2-μM Calcein AM, cell-permeant green dye (Invitrogen, Cat# C3100MP) and 0.2-μM ethidium homodimer-1 (Invitrogen, Cat# E1169) at 37°C for 10 min. Fluorescence images were acquired using an Olympus IX-83 inverted fluorescence microscope (Olympus Corporation, Tokyo, Japan; [RRID:SCR\\_020344](#)). Live and dead cells were quantified using the Fiji distribution of ImageJ (Open-source software, NIH, Bethesda, MD, USA, [RRID:SCR\\_002285](#)).

## 2.19 | Data and statistical analysis

The data and statistical analysis in this study comply with the *British Journal of Pharmacology* experimental design and analysis recommendations. All experiments were performed in at least three independent replicates unless otherwise stated. Data are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism Version 10. Concentration-response curves were fitted by nonlinear regression to derive IC<sub>50</sub> values. Comparisons among multiple groups were analysed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test. Post-hoc tests were run only if F achieved P < 0.05 and there was no significant variance inhomogeneity. Direct matched comparisons between TOL and ENT were analysed using a

paired two-tailed Student's *t*-test. Statistical significance was defined as  $P < 0.05$ . The exact replicate numbers, normalization procedures, and statistical tests are indicated in the corresponding figure legends.

## 2.20 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY <http://www.guidetopharmacology.org> and are permanently archived in the Concise Guide to PHARMACOLOGY 2025/26.

## 3 | RESULTS

### 3.1 | Tolcapone (TOL) and entacapone (ENT) suppress TAU aggregation kinetics with enhanced effects on mutant TAU

The effects of TOL and ENT on TAU aggregation were monitored using a ThT binding assay under conditions supporting spontaneous fibril formation, using WT R2R3 TAU (WT-R2R3) or P301S-mutant R2R3 TAU (P301S-R2R3) constructs. These peptide constructs represent a minimal aggregation-competent TAU model described previously (Das et al., 2025). Both TOL and ENT reduced aggregation of WT-R2R3 and P301S-R2R3 compared with untreated controls (Figure 1b,c). TEM of aggregation end-point samples showed abundant fibrillar assemblies under control conditions, whereas reactions performed in the presence of TOL or ENT contained markedly fewer and morphologically altered fibrillar structures (Figure 1d). Quantitative analysis showed that fibrils formed in the presence of either compound exhibited a shift towards reduced width distributions for both WT-R2R3f and P301S-R2R3f relative to control fibrils (Figure 1e,f).

To compare inhibitory efficacy across constructs and compounds, aggregation assays were performed over a range of TOL and ENT concentrations. Aggregation was quantified by calculating the AUC of ThT fluorescence kinetics for each condition and normalizing values to the corresponding untreated control, as described previously (Goud et al., 2025). Normalized aggregation decreased in a concentration-dependent manner for both WT-R2R3 and P301S-R2R3 in response to TOL or ENT (Figure 1g,h). Concentration-response curves were fitted using three-parameter logistic models to derive  $IC_{50}$  values for aggregation inhibition (Figure S1). These analyses revealed significantly lower  $IC_{50}$  values for P301S-R2R3 compared with WT-R2R3 for both compounds (Figure 1i,j). For TOL, the  $IC_{50}$  was  $13.12 \pm 4.12 \mu M$  for WT-R2R3 and  $1.32 \pm 0.28 \mu M$  for P301S-R2R3; for ENT, the corresponding values were  $24.91 \pm 4.84$  and  $2.34 \pm 0.49 \mu M$ , respectively.

### 3.2 | Tolcapone (TOL) and entacapone (ENT) bind P301S TAU more strongly than WT TAU

To test whether the preferential functional effects of TOL and ENT on mutant TAU reflect differential compound-TAU interactions, we quantified their binding to monomeric WT-R2R3 and P301S-R2R3 TAU constructs using SPR. Binding was concentration-dependent for both compounds across WT-R2R3 and P301S-R2R3 TAU, and global kinetic fitting yielded  $K_D$  together with  $k_{on}$  and  $k_{off}$  rate constants (Table 1 and Figure S2). TOL bound WT-R2R3 TAU with a higher  $K_D$  than P301S-R2R3 TAU, indicating stronger binding to the mutant. ENT displayed overall weaker binding than TOL but maintained a lower  $K_D$  for P301S-R2R3 than WT-R2R3.

To provide structural context, docking was performed using AlphaFold2-derived models of WT and P301S TAU (Figures 2 and S3). All structural models were evaluated for confidence and stereochemical quality prior to docking (Figures S4–S8). Docking identified defined binding regions for both ENT and TOL in WT and P301S TAU (Figure 2), with predicted binding scores in a comparable range across variants (Table S1). Consistent with the SPR measurements, predicted binding energies were modestly more favourable for P301S than for WT (Table S1). The additional model, P301L, included for comparative analysis, showed a similar binding pattern and energy range (Figure S8 and Table S1). Molecular dynamics-based binding analyses (Tables S2–S7) showed average MM-GBSA binding energies across 10 replicates that supported more favourable predicted interactions of ENT and TOL with mutant TAU (P301S and P301L) than with WT TAU.

### 3.3 | R2R3f formed in the presence of Tolcapone (TOL) and entacapone (ENT) show reduced pathogenicity in neuron-like SY5Y cells expressing TAUP301L-2N4R

To assess whether compound-modified TAU assemblies differ in their cellular pathogenicity, we examined their effects in SH-SY5Y cells expressing TAUP301L-2N4R-EGFP (SY5Y-TAUP301L). Differentiated cells were transduced with WT-R2R3f or P301S-R2R3f, formed either in the absence of compounds (control, CTL) or in the presence of TOL or ENT during fibril assembly, as described previously (Goud et al., 2025).

Immunoblot analysis of Triton X-100-insoluble fractions showed that seeding with WT-R2R3f or P301S-R2R3f generated under CTL conditions markedly increased levels of T22-reactive oligomeric and Ser262-phosphorylated TAU compared with buffer-only CTL (Figure 3a). In contrast, cells transduced with fibrils formed in the presence of TOL or ENT exhibited substantially reduced levels of both T22-positive TAU species and pTAU (Ser262) for both WT-R2R3f and P301S-R2R3f. Total TAU in the Triton X-100-insoluble fraction was

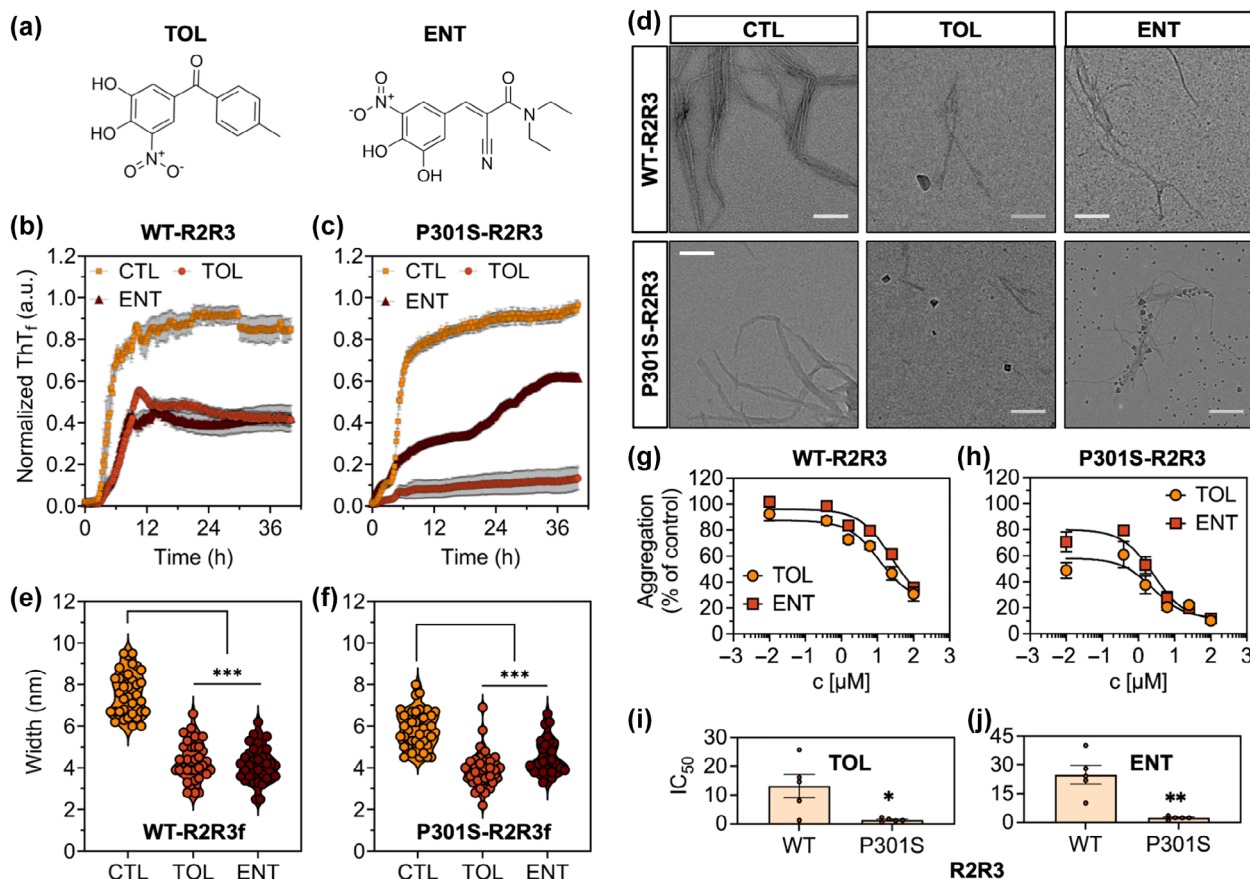


**FIGURE 1** Tolcapone (TOL) and entacapone (ENT) preferentially inhibit mutant TAU aggregation. (a) Chemical structures of TOL and ENT. ThT aggregation kinetics of WT-R2R3 (b) and P301S-R2R3 (c) TAU constructs incubated under standard aggregation conditions in the absence (control: CTL) or presence of 10-μM TOL or ENT. Curves represent mean  $\pm$  standard error of the mean (SEM) ( $n = 3$  independent experiments). (d) Transmission electron microscopy (TEM) images of aggregates formed from WT-R2R3 and P301S-R2R3 TAU constructs under CTL conditions or in the presence of TOL or ENT. Scale bar, 100 nm. Violin plots showing the distribution of fibril width measurements for WT-R2R3f (e) and P301S-R2R3f (f) formed under CTL conditions or in the presence of TOL or ENT. Each violin represents pooled measurements from multiple fibrils across two to three independent TEM images per condition; central lines indicate the median and interquartile range. \*\*\* $P < 0.001$ , one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test. Concentration-dependent inhibition of WT-R2R3 (g) and P301S-R2R3 (h) TAU aggregation by TOL and ENT, expressed as area under the curve (AUC) of ThT fluorescence normalized to the corresponding untreated control (aggregation, % of control). Data are mean  $\pm$  SEM ( $n = 5$  independent experiments). Corresponding ThT kinetic traces used for AUC calculations are shown in Figure S1. (i,j) IC<sub>50</sub> (μM) values for inhibition of aggregation of WT-R2R3 and P301S-R2R3 TAU by TOL and ENT. IC<sub>50</sub> values are derived from AUC-based concentration-response analyses of ThT fluorescence kinetics shown in Panels (g) and (h). Data are mean  $\pm$  SEM ( $n = 5$  independent experiments), \* $P < 0.05$ , \*\* $P < 0.01$ , two-tailed Student's  $t$  test (paired).

**TABLE 1** Surface plasmon resonance (SPR) kinetic parameters for Tolcapone (TOL) and entacapone (ENT).

|     | R2R3  | $K_D$ (M)             | $k_{on}$ ( $M^{-1}s^{-1}$ ) | $k_{off}$ ( $s^{-1}$ ) |
|-----|-------|-----------------------|-----------------------------|------------------------|
| TOL | WT    | $1.50 \times 10^{-7}$ | 53.24                       | $8.01 \times 10^{-6}$  |
|     | P301S | $3.30 \times 10^{-8}$ | 45.17                       | $1.49 \times 10^{-6}$  |
| ENT | WT    | $2.11 \times 10^{-4}$ | 7.431                       | $1.57 \times 10^{-4}$  |
|     | P301S | $1.25 \times 10^{-5}$ | 12.33                       | $1.54 \times 10^{-4}$  |

Note:  $K_D$ ,  $k_{on}$  and  $k_{off}$  values for TOL and ENT binding to WT-R2R3 and P301S-R2R3 TAU constructs, determined by surface plasmon resonance. Corresponding sensorgrams are shown in Figure S2. Lower  $K_D$  values indicate higher apparent binding affinity.

higher in cells seeded with control fibrils compared with buffer-treated cells (Figure 3a), while total TAU levels in the Triton X-100-soluble fraction remained unchanged across conditions (Figure S9).

Quantitative analysis confirmed significant reductions in T22-reactive oligomeric TAU and pTAU (Ser262) in cells seeded with WT-R2R3f or P301S-R2R3f formed in the presence of TOL or ENT compared with CTL fibrils (Figure 3b–e). Paired statistical analyses directly comparing the relative effects of the compounds showed that, for P301S-R2R3f-seeded cells, TOL-modified fibrils were associated with significantly lower levels of T22-positive oligomeric TAU and pTAU (Ser262) compared with ENT-modified fibrils (Figure S10).

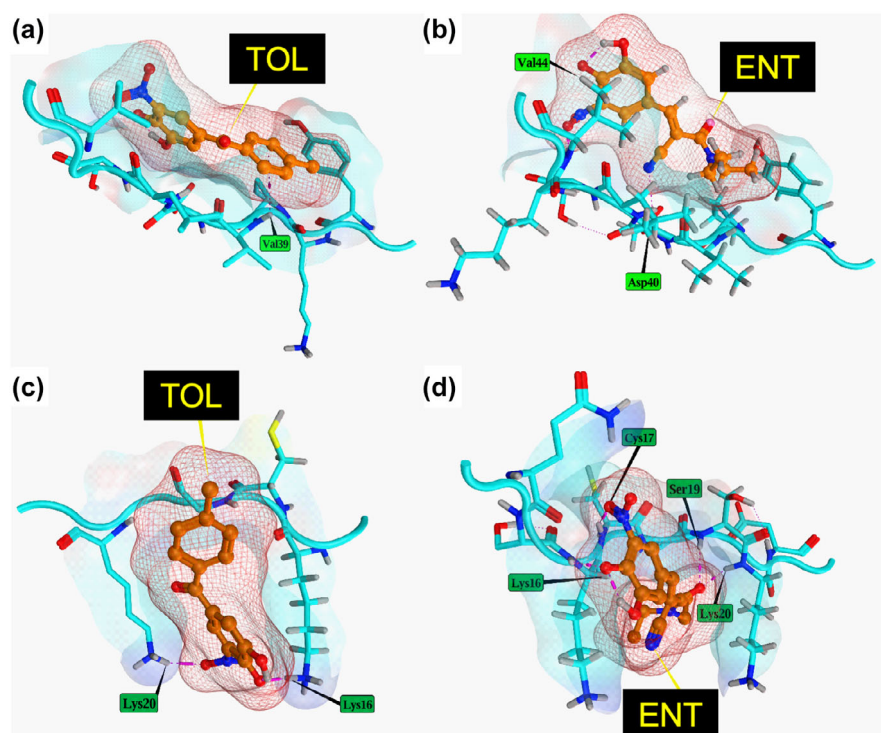


**FIGURE 2** Docking interactions of tolcapone (TOL) and entacapone (ENT) with wild-type (WT) and P301S tau filament models. Predicted binding poses of TOL (a, c) and ENT (b, d) docked to AlphaFold2-derived WT tau filaments (a, b) and P301S tau filaments (c, d). The tau filament is shown as a ribbon in surface representation, with selected interacting residues displayed as sticks. Ligands are represented as space-filling models coloured by atom type. Docking was performed using MOE 2024.01. Interactions are depicted as hydrogen bonds (yellow dashed lines), hydrophobic contacts (pink) and potential ionic interactions (violet lines).

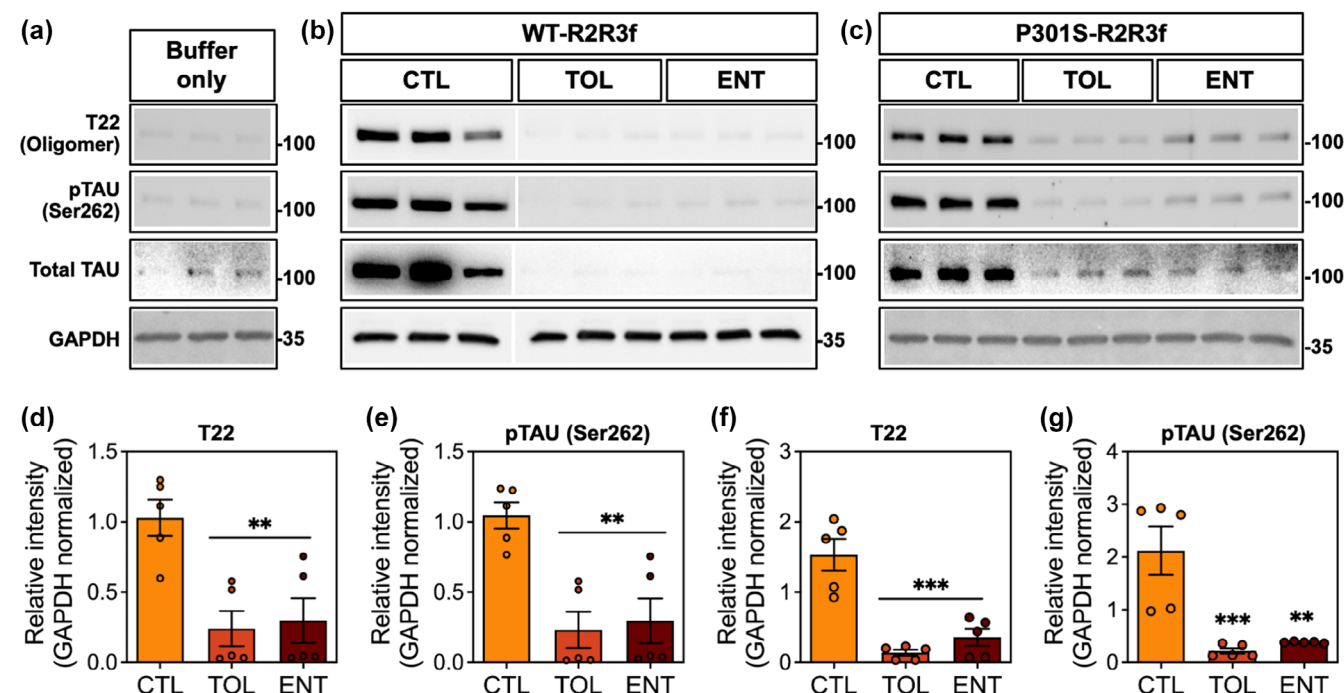


**FIGURE 3** Effect of tolcapone (TOL)- and entacapone (ENT)-modified R2R3f on TAU pathology in SY5Y-TAUP301L cells. (a–c) Western blot analysis of Triton X-100-insoluble fractions from differentiated SY5Y-TAUP301L cells treated with transfection buffer only (a) or transduced with WT-R2R3f (b) or P301S-R2R3f (c), formed in the absence of compounds (control: CTL) or in the presence of TOL or ENT (10 μM) during fibril formation. Representative blots of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from the soluble fraction are shown. Representative blots from three of five independent differentiation experiments are shown. Uncropped blots from all five experiments are provided in the supporting information. Images of uncropped blots are provided in the supporting information. (d–g) Quantification of TAU pathology in the Triton X-100-insoluble fraction. T22-positive oligomeric TAU (d, f) and pTAU (Ser262) (e, g) are shown for cells seeded with WT-R2R3f (d, e) or P301S-R2R3f (f, g) formed under control (CTL) conditions or in the presence of TOL or ENT during fibril formation. Data are mean ± standard error of the mean (SEM) (n = 5 independent differentiation experiments). \*\*P < 0.01, \*\*\*P < 0.001, one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test.

Differences between TOL and ENT were less pronounced for WT-R2R3f-seeded cells. Direct exposure of SH-SY5Y cells to TOL resulted in a concentration-dependent reduction in viability over 72 h, whereas ENT did not reduce viability below 50% at the highest concentration tested (Figure S11).

### 3.4 | Sonicated K18 fibrils are internalized by human induced pluripotent stem cell (hiPSC)-derived neurons and induce TAU aggregation and phosphorylation

To establish a TAU seeding paradigm in MAPT(P301L) isogenic hiPSC-derived neurons (hiPSC-iNs), Week 4 hiPSC(P301L)-iNs were exposed to sK18f labelled with Alexa Fluor 647 or to Alexa 647 dye alone (Figure 4a). Week 4 hiPSC(P301L)-iNs were previously characterized as mature and phenotypically stable (Wu et al., 2019). Twenty-four hours after exposure, intracellular 647-positive puncta were detected within the neuronal soma in neurons treated with sK18f-647 but not in dye-only controls (Figure 4b).

Following a three-week exposure to unlabeled sK18f, immunofluorescence analysis at Week 7 showed increased ProteoStat-positive puncta and elevated pTAU(S202/T205) immunoreactivity in sK18f-treated cultures compared with control (Figure 4c). ProteoStat-positive puncta were localized within the cytoplasm, and pTAU(S202/T205) signal was detected in TUJ1-positive neurons. Cultures showing increased ProteoStat puncta also exhibited overlapping pTAU(S202/T205) immunoreactivity.

### 3.5 | Tolcapone (TOL) and entacapone (ENT) modulate sarkosyl-insoluble TAU species in human induced pluripotent stem cell (hiPSC)-derived neurons

We next examined whether TOL or ENT modulates insoluble TAU species in neurons showing pre-existing TAU pathology. Sarkosyl fractionation followed by Western blot analysis was performed 3 weeks after sK18f exposure to assess TAU species in hiPSC(P301L)-derived neurons (Figure 5a). sK18f treatment increased levels of pTAU(S202/T205), pTAU(S262) and total TAU in the sarkosyl-insoluble fraction, while corresponding changes in whole-cell lysates were minimal (Figure 5b,c). LIVE/DEAD staining and LDH cytotoxicity assays showed no overt cytotoxicity in hiPSC-iNs treated with vehicle, TOL or ENT (Figure S13).

TOL or ENT was added 1 day after sK18f exposure and maintained throughout the incubation period, with thapsigargin applied during week 6 across all conditions as a uniform sensitizing background (Figure 5a). TOL treatment reduced sarkosyl-insoluble pTAU and total TAU in a dose-dependent manner, with the largest reduction observed at the highest concentration tested (Figure 5b,d). ENT treatment also reduced levels of insoluble TAU species, although the magnitude of change was consistently smaller across concentrations compared with TOL (Figure 5c,e). Quantitative normalization of band

intensities relative to unseeded vehicle controls confirmed that both TOL and ENT reduced pTAU(S262) and total TAU below the levels observed in the sK18f-seeded vehicle groups (Figure 5d,e). In both treatment conditions, TAU levels in whole-cell lysates remained largely unchanged.

### 3.6 | Tolcapone (TOL) and entacapone (ENT) suppress sK18f-induced TAU aggregation and phosphorylation in human induced pluripotent stem cell (hiPSC)(P301L)-derived neurons

Immunofluorescence analysis was performed in hiPSC(P301L)-derived neurons following sK18f seeding at Week 5 and subsequent compound treatment (Figure 6a). sK18f treatment increased intracellular protein aggregation and TAU phosphorylation, indicated by elevated ProteoStat staining and increased pTAU (S202/T205) immunoreactivity compared with unseeded CTLs (Figure 6b). Treatment with TOL or ENT reduced ProteoStat signal and pTAU (S202/T205) immunoreactivity relative to sK18f-treated neurons (Figure 6b).

Quantitative analysis showed a decrease in the number of ProteoStat<sup>+</sup>/DAPI<sup>+</sup> cells and a reduction in pTAU (S202/T205) fluorescence intensity following compound treatment, normalized to unseeded controls (Figure 6c,d). For TOL, a greater reduction in both readouts was observed at 50  $\mu$ M compared with 10  $\mu$ M (Figure 6c). ENT treatment also decreased ProteoStat positivity and pTAU (S202/T205) immunoreactivity relative to sK18f-treated neurons, with a smaller magnitude of change than that observed with TOL (Figure 6d).

## 4 | DISCUSSION

Tauopathies are driven by the accumulation and propagation of seed-competent TAU assemblies that template aggregation in recipient cells and promote the spread of pathology. Disease-associated MAPT mutations, particularly at residue P301, strongly enhance TAU aggregation and seeding, producing assemblies that differ structurally and functionally from WT TAU (Chen et al., 2019; Fischer et al., 2009). Identifying compounds that selectively target these pathogenic TAU conformers remains a key challenge in efforts to limit TAU-driven neurodegeneration.

In this study, we demonstrate that the clinically approved COMT inhibitors TOL and ENT suppress TAU aggregation in a concentration-dependent manner, exhibiting markedly greater potency against P301S mutant TAU than against WT TAU. Prior work has shown that catechol-containing compounds, including TOL and ENT, can inhibit the aggregation of TAU-derived peptides such as VQIVYK in cell-free systems, identifying the nitrocatechol moiety as a pharmacophore that can interfere with amyloid formation (Mohamed et al., 2013). However, those studies did not assess whether aggregation inhibition differs between WT and disease-associated mutant TAU conformers. Our findings extend this earlier work by providing evidence that

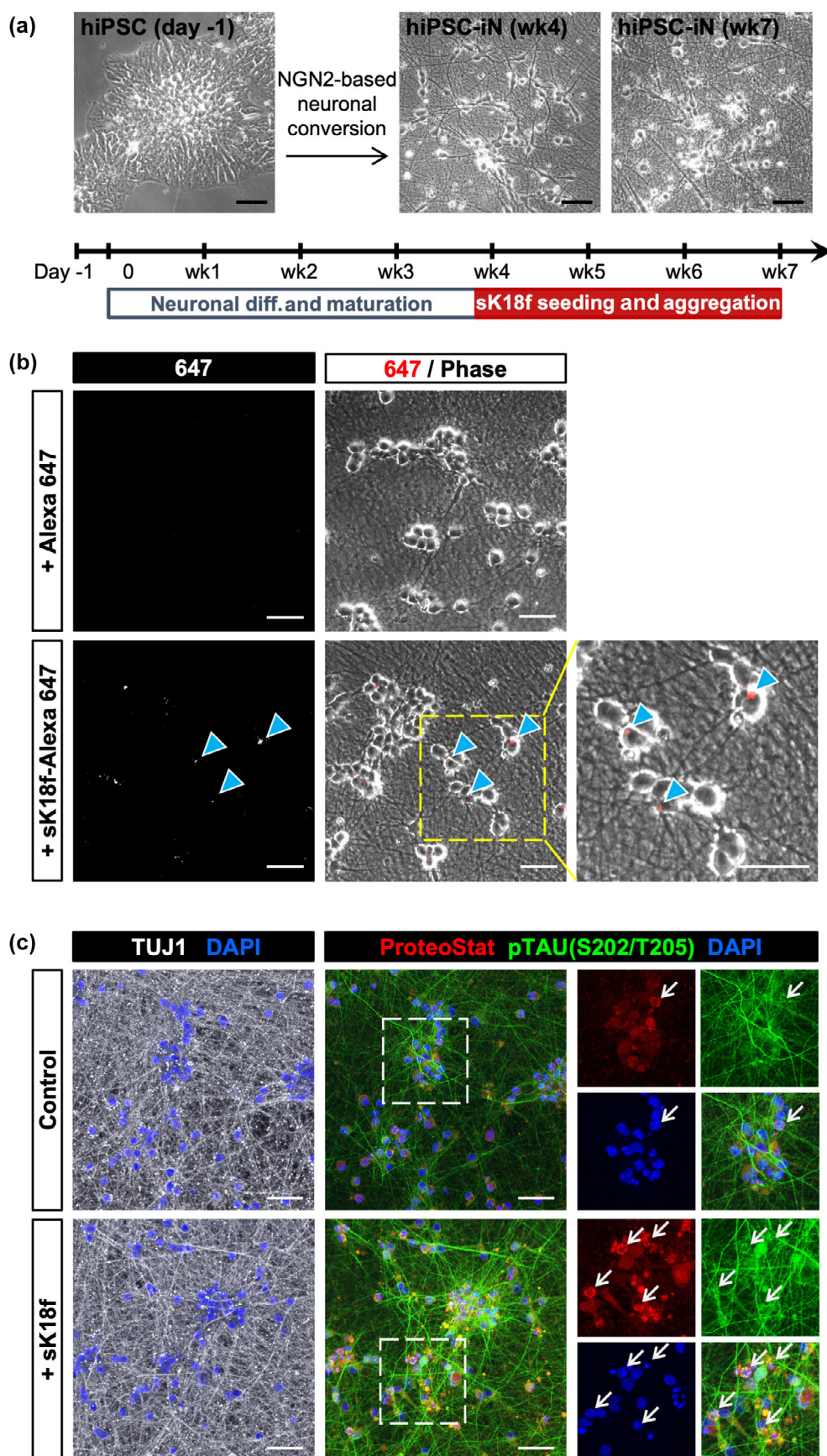


**FIGURE 4** sK18 fibrils enter human induced pluripotent stem cell (hiPSC)-derived neurons and induce TAU aggregation.

(a) Schematic of the experimental timeline showing NGN2-driven neuronal differentiation of hiPSCs and subsequent sK18f seeding phase (Weeks 4–7). Representative phase-contrast images show the morphology of iPSCs, Week 4 induced neurons, and Week 7 mature neurons. Scale bar, 50  $\mu$ m. (b) Alexa Fluor 647-labelled sK18 fibrils (sK18f-647) or Alexa Fluor 647 control dye were delivered into Week 4 hiPSC-iNs using Xfect transfection. Images were acquired 24 h after transfection. The 647-fluorescence channel and merged 647/phase images are shown. Cyan arrowheads indicate 647-positive puncta within the dashed yellow box, with the corresponding high-magnification view displayed on the right. Scale bar, 50  $\mu$ m. (c) hiPSC(P301L)-iNs were fixed at Week 7 and immunostained for TUJ1, ProteoStat, pTAU(S202/T205) and DAPI. Full-field images of control and sK18f-treated cultures are shown. Dashed boxes indicate regions presented at higher magnification on the right, displaying individual channels for ProteoStat, pTAU(S202/T205) and DAPI. White arrows mark cells containing ProteoStat-positive puncta. Scale bar, 50  $\mu$ m.

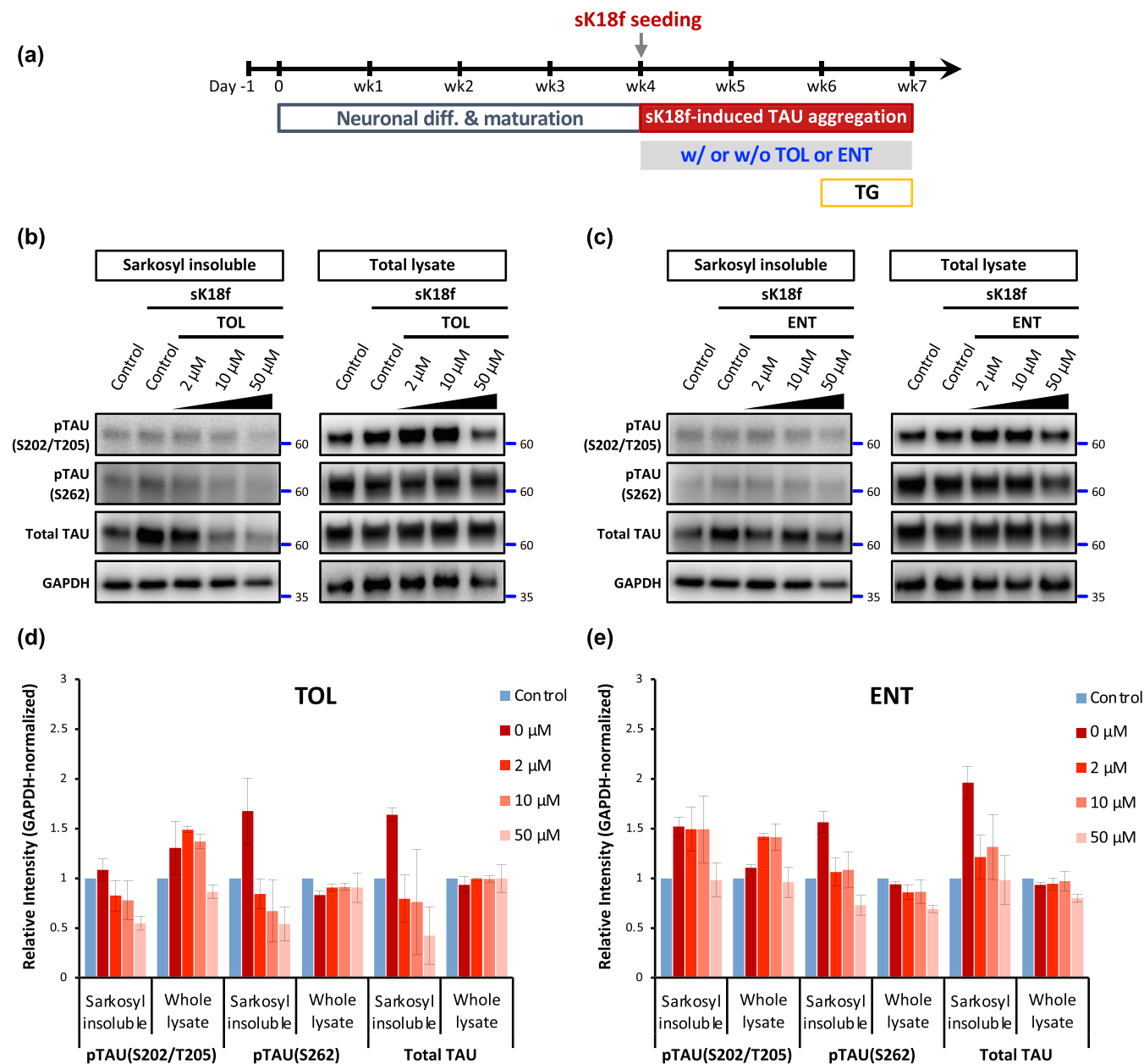


**FIGURE 5** Effects of tolcapone (TOL) and entacapone (ENT) on sarkosyl-insoluble TAU species in human induced pluripotent stem cell (hiPSC)(P301L)-derived neurons. (a) Experimental workflow showing the timeline of NGN2-induced neuronal differentiation, sK18f seeding and treatment with TOL or ENT. Compounds were added 1 day after sK18f exposure and maintained for 3 weeks. Thapsigargin (TG) was applied for 1 week at Week 6. (b, c) Western blots of pTAU(S202/T205), pTAU(S262), total TAU and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in sarkosyl-insoluble fractions and total lysates from neurons treated with sK18f in the presence or absence of TOL (b) or ENT (c) at 2, 10 or 50  $\mu$ M. Images of uncropped blots are provided in the [supporting information](#). (d, e) Quantification of GAPDH-normalized TAU signals in the sarkosyl-insoluble fraction and whole lysate corresponding to Panels (b) and (c). Data are mean  $\pm$  standard error of the mean (SEM) ( $n = 3$  biological repeats). Where  $n < 5$  experiments, statistical analysis was not carried out, and results should be regarded as preliminary.

catechol-based inhibition of TAU aggregation differs between WT and P301-mutant TAU under the experimental conditions used. This aligns with structural evidence that P301 mutations destabilize the local architecture at the R2–R3 interface, thereby increasing the exposure of amyloidogenic motifs (Chen et al., 2019; Mirbaha et al., 2018).

Importantly, the suppression of bulk TAU aggregation does not necessarily translate into reduced TAU propagation. Therefore, it is

critical to determine whether compounds that inhibit fibril formation also reduce the pathogenic activity of TAU assemblies in cellular systems. Our findings address a major limitation of many TAU aggregation inhibitors, which successfully reduce fibril formation in vitro but fail to block the seed-competent TAU species that actually drive the spread of pathology (Hill et al., 2022; Soeda et al., 2019; Soeda & Takashima, 2020).

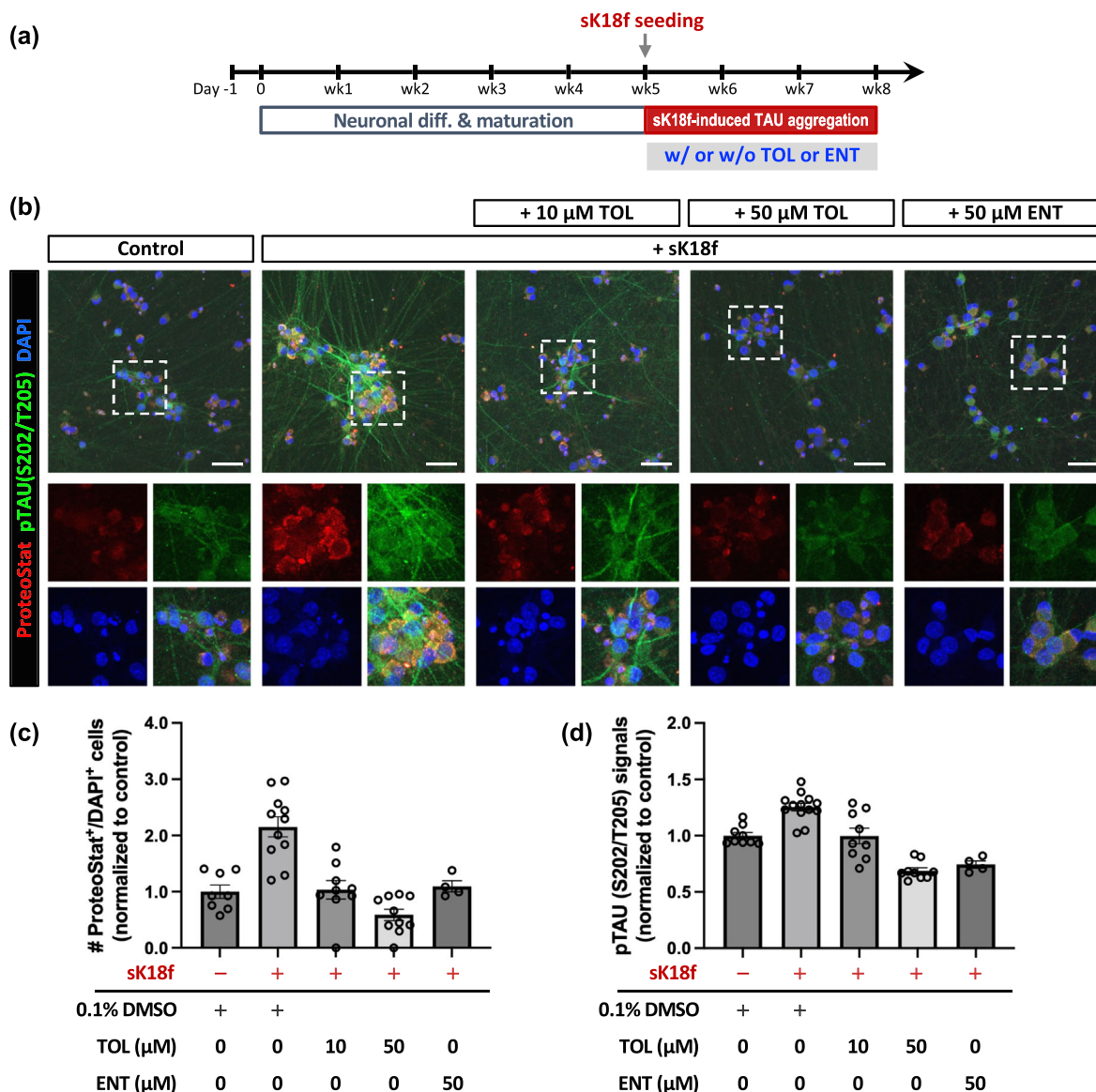


**FIGURE 6** Tolcapone (TOL) and entacapone (ENT) reduce sk18f-induced TAU aggregation and phosphorylation in hiPSC(P301L)-derived neurons. (a) Timeline of sk18f seeding and drug treatment. TOL or ENT was added 1 day after sk18f exposure and maintained for 3 weeks. (b) Immunofluorescence of hiPSC-iNs fixed at Week 8 under control conditions, sk18f treatment or sk18f plus TOL (10 or 50  $\mu$ M) or ENT (50  $\mu$ M). Dashed boxes denote regions shown in magnified insets. Scale bar, 50  $\mu$ m. (c) ProteoStat<sup>+</sup>/DAPI<sup>+</sup> cell counts normalized to control for TOL or ENT treatment conditions. Data are mean  $\pm$  standard error of the mean (SEM) from two independent differentiation and treatment experiments, with 4–12 imaging fields analysed per condition within each experiment. (d) pTAU(S202/T205) fluorescence intensity normalized to control for TOL or ENT treatment conditions. Data are mean  $\pm$  SEM ( $n = 4$ –12 imaging fields from two independent differentiation and treatment experiments). Where  $n < 5$  experiments, statistical analysis was not carried out, and results should be regarded as preliminary.

SPR analysis showed that both TOL and ENT exhibited higher apparent binding affinity for monomeric P301S-R2R3 TAU than WT-R2R3 TAU, providing a biochemical correlation with their preferential functional effects on mutant TAU. Consistent with the SPR measurements, docking and molecular dynamics analyses predicted differential interactions with mutant and WT TAU. However, these binding and computational data do not establish the molecular mechanism responsible for aggregation or seeding inhibition. In particular, they cannot determine whether the compounds act primarily through monomeric TAU, transient aggregation intermediates, oligomeric species or

redirection of the aggregation pathway. The SPR and computational findings should therefore be considered supportive mechanistic evidence rather than a direct demonstration of the functionally relevant binding event.

SPR-derived affinities to monomeric TAU do not necessarily predict functional potency in aggregation or seeding assays (Chai et al., 2024; Schafer et al., 2013). High-resolution cryo-electron microscopy studies have shown that pathogenic TAU fibrils contain a conserved core centred on the VQIVYK motif (Falcon et al., 2018; Fitzpatrick et al., 2017). The higher apparent affinity for mutant TAU

may therefore reflect increased accessibility of aggregation-prone interaction surfaces, although this interpretation remains speculative and requires direct analysis of compound interaction with aggregation intermediates and fibrillar species.

The functional relevance of these findings was further supported in neuronal models. In SY5Y-TAUP301L cells, fibrils formed in the presence of TOL or ENT induced lower levels of TAU oligomerization and Ser262 phosphorylation, indicating reduced pathogenic activity of the resulting assemblies. By testing the compounds during fibril formation, this experiment demonstrates that drug-modified TAU assemblies exhibit reduced pathogenic capacity upon entering recipient cells. Catechol-based compounds have previously been reported to reduce TAU oligomerization and pathological TAU accumulation in cellular systems and transgenic mouse models through cysteine-dependent mechanisms (Soeda et al., 2015). However, those studies focused primarily on early oligomer formation and overall pathology burden, rather than directly examining seed competence or post-seeding propagation. Our results extend this work by demonstrating that catechol-containing compounds can reduce the downstream pathological impact of TAU assemblies with defined seeding histories.

Extending these observations to a human neuronal context, we leveraged human iPSC-derived neuronal seeding models to test translational relevance. We showed that pathogenic K18 fibrils are efficiently internalized by hiPSC-derived neurons, inducing robust TAU aggregation and phosphorylation that recapitulate key features of tauopathy (Goedert & Spillantini, 2017). In this system, TOL and ENT were applied after fibril exposure to determine whether these compounds can suppress intracellular TAU pathology once seeding has been initiated. Treatment with TOL, and to a lesser extent ENT, reduced sarkosyl-insoluble TAU species and cellular markers of aggregation and phosphorylation. Notably, we incorporated orthogonal viability controls (LIVE/DEAD staining and LDH release) under vehicle-matched conditions, supporting that the observed reductions in seeded TAU readouts are not readily explained by overt cytotoxicity. The consistency of compound effects across biochemical assays, cellular models and human iPSC-derived neurons, particularly in P301 mutant contexts, supports a model in which TOL and ENT preferentially interfere with mutation-driven, seed-competent TAU assemblies.

From a translational perspective, TOL crosses the blood-brain barrier and inhibits COMT centrally, whereas ENT acts primarily as a peripheral COMT inhibitor with limited brain penetration (Lees, 2008). However, the clearest effects in the hiPSC-derived neuronal model were observed at concentrations up to 50  $\mu$ M, which are higher than the clinically relevant free CNS exposures expected for these compounds. The present experiments, therefore, demonstrate pharmacological activity under controlled cellular conditions but do not establish that either marketed drug can achieve sufficient brain exposure to reproduce these effects clinically. This limitation is particularly important for ENT because of its restricted CNS penetration and for TOL because its known hepatotoxicity constrains chronic administration and dose escalation (Artusi et al., 2021). Accordingly, our findings should not be interpreted as supporting immediate clinical repurposing of TOL or ENT. Rather, they identify catechol-based scaffolds as

starting points for developing more potent TAU-directed analogs with improved CNS exposure and safety.

This study is not without limitations. First, the human iPSC-neuronal experiments were performed using a single MAPT(P301L) isogenic clone. Although all treatment comparisons were conducted within the same genetic background, clone-specific effects cannot be excluded. The hiPSC-derived neuronal findings should therefore be regarded as preliminary proof-of-concept evidence and require replication in independently edited clones or patient-derived lines before they can be generalized. Second, some experimental datasets were performed using three independent replicates, which limits statistical power and the precision of the estimated effects. Third, the SPR measurements were performed using monomeric R2R3 TAU constructs and therefore do not identify the functionally relevant TAU species or establish the molecular mechanism of aggregation and seeding inhibition. Fourth, the concentrations producing the strongest effects in hiPSC-derived neurons exceed the clinically relevant free CNS exposures expected for the marketed compounds. Finally, future studies should evaluate optimized analogs with improved potency, CNS exposure and safety in additional human neuronal models, 3D systems and animal models.

In conclusion, TOL and ENT preferentially reduced the aggregation and downstream pathogenic activity of P301-mutant TAU in the biochemical and cellular models examined in this study. The consistency of these effects across complementary assays supports further investigation of catechol-based compounds as modulators of seed-competent TAU assemblies. However, the use of a single isogenic hiPSC clone, limited replication in selected datasets, uncertainty regarding the relationship between monomer binding and functional inhibition and the disparity between effective cellular concentrations and clinically relevant CNS exposure preclude conclusions regarding direct therapeutic repurposing. These findings instead provide proof-of-concept support for the development of more potent and safer TAU-directed analogs with improved brain exposure.

## AUTHOR CONTRIBUTIONS

**Ihor Kozlov:** Investigation; methodology; writing—original draft; formal analysis. **Yu-Sheng Hung:** Methodology; investigation; formal analysis; writing—original draft. **Sudeep Roy:** Writing—original draft; visualization; formal analysis; investigation. **Alladi Charanraj Goud:** Formal analysis; investigation; methodology. **Roman Kouřil:** Investigation; methodology. **Yu-Hui Wong:** Writing—original draft; formal analysis; supervision; writing—review and editing; funding acquisition. **Viswanath Das:** Conceptualization; funding acquisition; writing—original draft; writing—review and editing; formal analysis; supervision; project administration.

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## CONFLICT OF INTEREST STATEMENT

The authors have no relevant financial or non-financial interests to disclose.

## DATA AVAILABILITY STATEMENT

All data supporting the findings of this study are provided within the article and its supporting information. In addition, all raw and processed datasets are publicly available at Zenodo (DOI: <https://doi.org/10.5281/zenodo.18618834>). The repository includes raw ThT kinetic traces, TEM fibril width measurements, ImageJ macros, global kinetic-fitting analyses and complete MM-GBSA and per-residue energy-decomposition outputs.

## DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for [Design and Analysis](#) and [Immunoblotting and Immunochemistry](#), where applicable, and as recommended by funding agencies, publishers, and other organizations engaged with supporting research. No animal experiments were performed in this study; therefore, [ARRIVE reporting guidelines](#) were not applicable.

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## SUPPORTING INFORMATION

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