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Drug quantification in single 3D organoids reveals correlation to reported in vivo brain uptake and demonstrates translational relevance for prediction of blood-brain barrier permeability

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Research Article

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Drug quantification in single 3D organoids reveals correlation to reported *in vivo* brain uptake and demonstrates translational relevance for prediction of blood-brain barrier permeability

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Abstract:

Evaluation of blood-brain barrier (BBB) permeability is a crucial step in drug research and development, particularly for the pharmacotherapy of targets in the central nervous system. Current preclinical permeability predictions are hindered by limited transferability or BBB model inaccessibility for analysis. In order to generate relevant pharmacological data, it is necessary to establish qualified physiological, translational, and quantitative systems with validated methods.

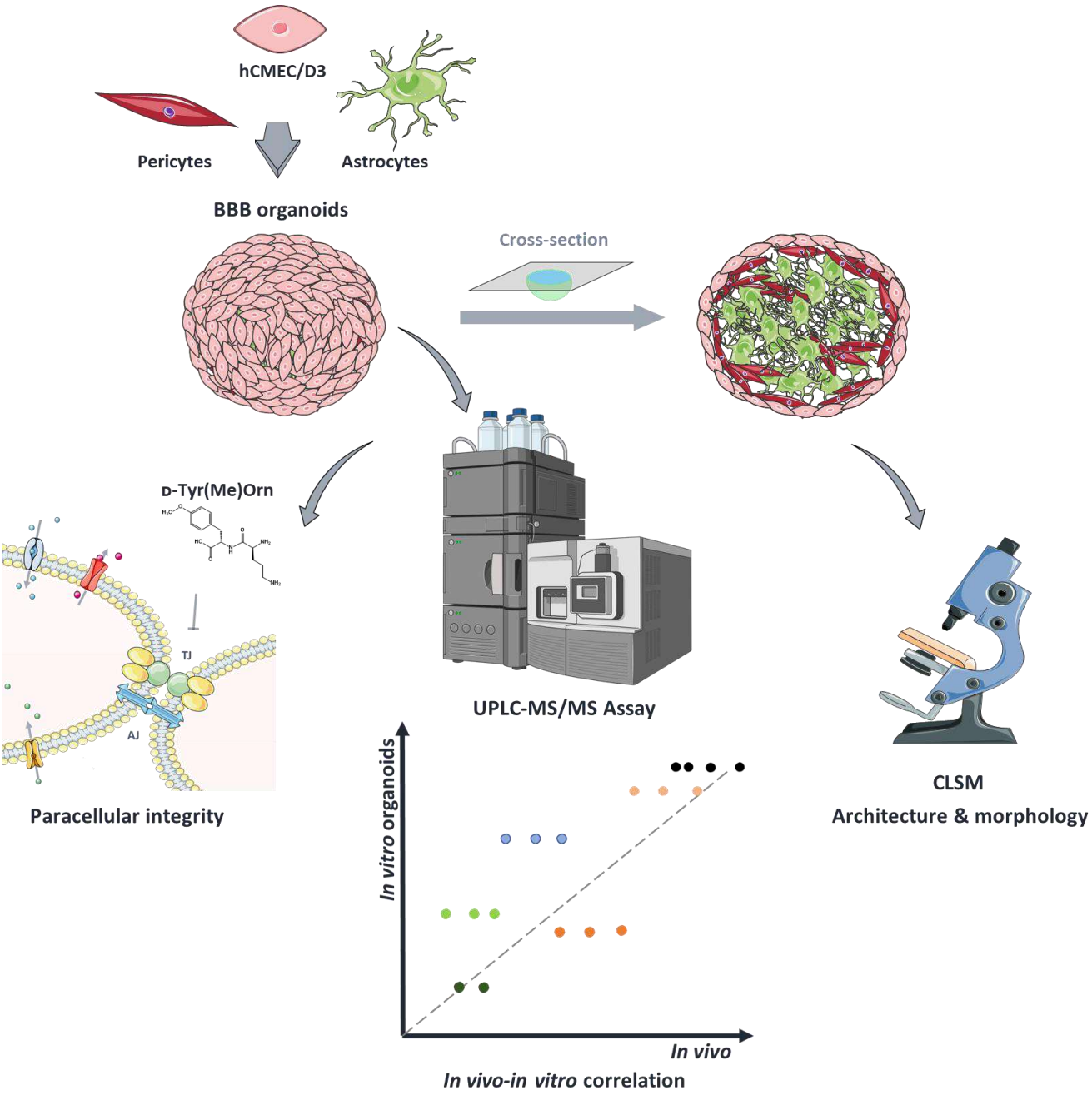
3D BBB organoids composed of human cerebral microvascular endothelial cells (hCMEC/D3), and primary pericytes and astrocytes were generated. The BBB barrier function was evaluated using the paracellular marker D-Tyr(Me)Orn. Confocal laser scanning microscopy was used to determine the architecture of the cell arrangements, crucial BBB hallmarks and the apoptosis rate (cleaved caspase 3). An experimental setup was established to evaluate drug uptake into the organoids using ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS). The concentration- and time-dependent intraorganoidal drug uptake was determined for 13 drugs, and used to calculate the ratio of intra- and extraorganoidal concentrations. These organoid uptake ratios were subsequently correlated with reported *in vivo* brain-to-plasma ratios. Additionally, the performance of four *in silico* BBB permeability prediction tools was assessed with the *in vivo* data.

Optimal analysis time point was identified as day 3 post self-assembly based on organoid architecture, endothelial coverage and apoptosis rate. Modular barrier properties were shown by a reduction of intraorganoidal D-Tyr(Me)Orn after cell connection were compromised, and potential wash out cross-validated by fluorescent Cy5 in the wash solution. Deming regression demonstrated a correlation with pseudo R^2 of 0.64 between the organoid uptake ratios and reported mammalian *in vivo* brain-to-plasma ratios. In contrast, the *in silico* tools evaluated displayed no correlations with these *in vivo* data.

We established and validated a simple and rapid experimental setup for the UPLC-MS/MS drug quantification into single 3D BBB organoids. We demonstrated that the 3D BBB organoids have translational relevance with regard to human and mammalian *in vivo* BBB permeability. This system therefore qualifies as New Approach Methodology (NAM) and can be used for approximate evaluation of *in vivo* brain uptake, while outperforming state of the art *in silico* models.

Keywords: 3D blood-brain barrier organoids; blood-brain barrier; paracellular marker; permeability; liquid chromatography tandem mass spectrometry; drug screening, New Approach Methodologies.

Graphical abstract:



Background

The translational relevant evaluation of blood-brain barrier (BBB) permeability in preclinical models is essential for the development of drug candidates, especially for central nervous system (CNS) pathologies. Because the BBB is highly restrictive, the majority of drugs reach minimal exposure in the CNS[1,2]. Currently, many CNS pathologies are still not accessible to efficacious therapeutic options. Although the BBB can be impaired in pathological states such as brain tumors, it can still prevent an effective drug treatment of CNS malignancies. Brain tumors are a type of CNS diseases with a particularly poor prognosis, and they can result in a less restrictive blood-tumor barrier (BTB). But effective pharmacotherapy requires drug access to all cancer cells to prevent relapse. This includes infiltrating cells that are farther away from the BTB and metastases that can often localize behind an intact BBB[3,4].

The scarcity of human BBB permeability data is mainly caused by limited accessibility and invasive sampling as a major hurdle for clinical monitoring of CNS drug exposure. While animal data is abundant, it suffers from the usual limitations of species differences in particular with regard to receptor and transporter mediated uptake and efflux, resulting in remaining uncertainty for clinical efficacy or safety[5-12]. In addition, limiting the use of animals for preclinical research is a main goal for ethically responsible research, with extensive general regulatory support[13-15].

Studies in animals have demonstrated limited predictability of human drug safety and efficacy reflected by the high attrition rate of clinical candidates evaluated with animal toxicological studies[15,16]. In context of BBB permeability, this is especially evident for P-glycoprotein (P-gp) substrates in rodents[5-10]. Many alternative *in vitro* models have historically relied on cell lines and suffer from similar or more pronounced drawbacks of animal experiments in regard to translational relevance[17,18]. The huge variety of current *in vitro* BBB models represents a tradeoff between the physiological replication of the complex BBB architecture and function and accessibility for analysis. Nevertheless, *in vitro* models have decisive advantages. They offer a much higher throughput with lower resource demand and, importantly, help to reduce the number of laboratory animals (e.g. 3R principle: reduce, replace, refine[14]). At the same time, they can be composed entirely of human cells, thus circumventing species differences. Translationally relevant models are therefore urgently required to accelerate drug

development. With the rise of New Approach Methodologies (NAMs) and their recent acceptance in toxicological testing by the FDA[15], drug development is undergoing a silent revolution. NAM include human primary cells, induced pluripotent stem cell (iPS)-derived cells, computational models, AI-predictions, organ-on-a-chip, and organoids. Moving to human-centric testing promises more relevant insights into human physiological and biological response, and mechanisms of toxicity. A key prerequisite of NAM use in regulatory preclinical testing is their qualification consisting of the demonstration of their reliability, reproducibility, and the fit-for-purpose. Especially in the context of the BBB, organoid models, like other NAMs, are essential for achieving the interaction between astrocytes, pericytes and endothelial cells that facilitates the most restrictive barrier in human physiology.

We aimed to establish a NAM model for BBB permeability and qualify its translational relevance. We selected a simple self-assembling 3D organoid[19,20] that allows direct interaction of endothelial cells, astrocytes, and pericytes as the key BBB architecture. While brain capillary endothelial cells (BCEC), are the main BBB component, the additional cell types have been shown to contribute significantly to BBB establishment and maintenance *in vivo*[21-32] and to the BBB phenotype of endothelial cells *in vitro*[33-47]. These organoids are easy to reproduce and comparatively cost-efficient. They are fully human and the cell types used (hCMEC/D3 and primary astrocytes and primary pericytes) are readily available. In the 3D BBB organoid astrocytes and pericytes can interact freely and are surrounded by a continuous layer of the BCEC hCMEC/D3 cells[20]. Endothelial cells of these organoids express crucial BBB hallmarks, such as Zonula occludens 1 (ZO-1), claudin-5, low density lipoprotein receptor-related protein 1 (LPR-1), the transferrin receptor, and P-gp[20,48]. P-gp-mediated transport was demonstrated to be functionally active[20,49], as was the transcytosis mediated by the transferrin and LRP-1 receptors[20,48]. This model has been used for targeting construct assessment, antibody uptake[20,48] and toxicology screening[49]. As an initial proof-of-concept, the penetration of two drugs was qualitatively evaluated with matrix-assisted laser desorption/ionization (MALDI) imaging[19,20]. The presence of a functional barrier was previously mainly demonstrated by increased high molecular marker uptake after tight junction (TJ) opening, like dextran uptake after VEGF treatment[20], which is a limited discriminative marker for the maximal restrictive BBB based on its large size.

Our ambition was the development of a translationally qualified permeability methodology based on the optimization of these NAM organoid culture establishing a high-throughput analysis of drug uptake on single organoid level via highly sensitive ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) measurements. For this purpose, we investigated the uptake of the validated highly discriminative paracellular marker D-Tyr(Me)Orn[50] and 13 drug (candidates) with known brain concentrations *in vivo* in the context of emerging therapies for brain tumor treatment. Our aim was to establish a NAM for BBB permeability. Therefore, our main objective was to evaluate the *in-vitro-in-vivo* correlation and thereby the translational relevance of the organoid model for the prediction of human BBB permeability.

Methods

Materials

hCMEC/D3 were purchased from Cellutions Biosystems Inc. (Burlington, Canada), normal hippocampal primary astrocytes from Innoprot (Bizkaia, Spain) and primary human vascular pericytes and Pericyte Medium (PM) from ScienCell Research Laboratories (Carlsbad, CA, USA). Endothelial Growth Medium 2 SingleQuots™ (EGM-2™) and Astrocyte Growth Medium (AGM™) were provided by Lonza (Basle, Switzerland). Roswell Park Memorial Institute (RPMI) 1640 medium was obtained from PAN Biotech (Aidenbach, Germany). Cell+ flasks with vented caps and standard cell culture flasks were purchased from Sarstedt (Nuremberg, Germany).

Akura™ 96 spheroid microplates were obtained from Insphero (Schlieren, Switzerland). Antibodies were obtained as followed: mouse anti-human ZO-1 (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), mouse anti-human P-gp (BD Biosciences, Heidelberg, Germany), mouse anti-human CD31 ab9498 (Dako, Santa Clara, CA, USA), rabbit anti-active caspase 3 (R&D Systems, Minneapolis, MN, USA); goat anti-mouse Alexa-Fluor® 488 secondary antibody (Abcam, Cambridge, UK) goat anti-rabbit Alexa-Fluor® 488 secondary antibody, anti-mouse Alexa-Fluor® 647 secondary antibody (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). CellTracker™ Deep Red was also purchased from Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA). Penicillin-streptomycin solution, Dulbecco's phosphate-buffered saline (PBS), Hanks Balanced Salt Solution (HBSS), trypsin-ethylenediamine tetraacetic acid (EDTA) solution (0.5 g porcine trypsin and 0.2 g EDTA/L), fetal bovine serum (FBS), 1 M HEPES buffer, lactate dehydrogenase (LDH) assay, dimethyl sulfoxide (DMSO), Fluoroshield™ with DAPI, adenosine, Triton X-100, human serum (male AB) and collagenase IV were purchased from Sigma-Aldrich (Taufkirchen, Germany). Ethylene glycol tetraacetic acid (EGTA) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). HumanKine® recombinant human vascular endothelial growth factor (VEGF)165 protein was purchased from Proteintech (Planegg-Martinsried, Germany). Tert-butyl methyl ether (TBME) and the ammonia solution (NH_4OH, aqueous 28%) were provided by Merck (Darmstadt, Germany). CellTiter-Glo® 3D Cell Viability Assay was obtained from Promega (Madison, USA). Human albumin 20% was obtained from CSL Behring (Marburg, Germany). Bovine albumin fraction V (BSA) and Tween®-20

were purchased from AppliChem (Darmstadt, Germany). The acetonitrile (ACN), and formic acid (FA) were from Biosolve (Valkenswaard, The Netherlands) all at highest analytical purity. Ultra-purified water was produced with the Arium® mini water system (Sartorius, Göttingen, Germany). Hydrochloric acid (HCl) and sodium hydroxide (NaOH) and were provided by Honeywell (Seelze, Germany). Repotrectinib, futibatinib, lorlatinib, and larotrectinib were purchased from MedKoo Biosciences (Durham, USA); [$^{2}H_6$]-futibatinib, [$^{2}H_3$,$^{13}C_1$]-lorlatinib, [$^{2}H_4$]-larotrectinib, temsirolismus and [$^{13}C_3$, $^{2}H_7$]-temsirolimus were purchased from AlsaChim Shimadzu, (Illkirch-Graffenstaden, France); ulixertinib and [$^{2}H_6$]-ulixertinib were obtained from BioMedValley, Asymchem Life Science, (Tianjin, China); olaparib and [$^{2}H_4$]-olaparib were purchased from Biozol GmbH (Hamburg, Germany). Selinexor was obtained from Selleckchem (Cologne, Germany), hypericin from Dr. Willmar Schwabe Pharmaceuticals (Karlsruhe, Germany), fluoxetine from Sulpelco Sigma-Aldrich (Taufkirchen, Germany), [$^{2}H_6$]-fluoxetine from Cerilliant (Round Rock, TX, USA), octreotide from BACHEM (Bubendorf, Switzerland), [$^{13}C_6$]-octreotide from TRC (Vaughan, ON, Canada), paroxetine from EDQM (Strasbourg, France), diazepam from Serva Feinbiochemica (Heidelberg, Germany), senicapoc from MedChemExpress (Monmouth Junction, NJ, USA) and selitrectinib from Hycultec GmbH (Beutelsbach, Germany).

The synthesis of D-Tyr(Me)Orn and the stable isotopically labeled D-Tyr([$^{2}H_3$]Me)Orn has been reported previously[50].

Stock solutions and dilutions

The drugs were dissolved in PBS with a minimal concentration of DMSO when necessary (generally < 0.1% except for highest concentration of selitrectinib with 1.31% and olaparib with 0.15%). D-Tyr(Me)Orn was stored in a 40 mM stock solution in PBS. All working solutions were freshly prepared after thawing on ice as described in the respective experimental section. The EGTA solution (5 mM) was prepared by diluting the 10 mM EGTA powder in PBS and adjusting the PH to ~ 8 and subsequent dilution in cell culture medium. The 1:10 dilution of trypsin was performed in PBS. Lyophilized VEGF was dissolved in PBS with 1% human serum albumin to a concentration of 100 µg/mL. Adenosine was dissolved in PBS to a concentration of 40 mM. Collagenase IV was dissolved in PBS to a concentration of 15 mg/mL and frozen in aliquots. For organoid lysis, the collagenase IV was thawed and diluted

178 further to 3 mg/mL in PBS. All stock solutions were prepared in their respective solvents and stored in
179 appropriate aliquots at -20 °C.

180 **Cell culture and *in vitro* experiments**

181 hCMEC/D3 cells, primary human astrocytes and pericytes were cultured separately in Cell+ culture
182 flasks in a CO_2 incubator at 37 °C and with 5% CO_2. The hCMEC/D3 cells were kept in EGM-2 medium,
183 the astrocytes in AGM medium and the pericytes in PM medium, each medium mixed with all respective
184 supplements. The cells were washed with HHBSS and detached with trypsin solution for the minimum
185 required time. Detachment was stopped by adding RPMI medium supplemented with a penicillin-
186 streptomycin solution (100 U/mL and 100 µg/mL) and 10% FBS. hCMEC/D3 were used until and
187 including passage 35, astrocytes until passage 5 and pericytes until passage 10 (as previously reported[19]).

188 **Organoid generation**

189 Organoids were generated as described by[19], with the following adaption: 1,500 of each cell type,
190 counted using the MACSQuant® Analyzer (Miltenyi Biotec), were transferred to one well of an Akura™
191 spheroid microplate for self-assembly (day 0). To reduce smaller secondary organoids, the medium was
192 changed after 24 h (day 1). Afterwards, gentle rotation on the rotation shaker in the CO_2 incubator was
193 applied for the remaining culture duration to induce shear stress through flow. Unless otherwise stated,
194 all experiments were performed on day 3 after assembly. As previously published, the organoids were
195 cultured in organoid medium (EGM-2 without VEGF and FBS, supplemented with 2% human serum).
196 This resulted in one organoid per well of the spheroid plate.

197 **Staining and immunofluorescence**

198 For CellTracker™ staining, the described procedure[20] was adapted as follows: cells were stained in the
199 cell culture flask with 5 µM CellTracker™ solution in HHBSS before detachment. After a 15-min
200 incubation at 37 °C, the cells were washed twice with warm HHBSS, detached with trypsin solution and
201 centrifuged. The cells were then gently resuspended in the organoid medium and used for organoid
202 generation. After the respective culture time, the organoids were fixed for 1 h with a 4% formaldehyde
203 solution.

204 For separate or combined immunostaining, the Stemcell™ Technologies protocol „performing
205 immunocytochemical staining of epithelial organoids“ was used for orientation. All incubation steps

were performed at room temperature on an orbital shaker and all washing steps lasted 5 min. The organoids were pooled in low-adherence reaction tubes. After formaldehyde fixation, the organoids were incubated for 30 min in a 0.3 M glycine solution and washed three times with an immunofluorescence-buffer (IF-buffer, 0.1% BSA (w/v), 0.2% Triton-X-100 (v/v), 0.05% Tween 20 (v/v) in PBS). The organoids were either directly permeabilized and blocked (5% BSA (w/v), 1% Triton-X-100 (v/v) in PBS) overnight or stored in PBS at 4 °C. After permeabilization staining with the respective primary antibody was performed overnight. The antibodies (CD31, ZO-1, P-gp, Cas3) were diluted 1:100 in IF buffer. After 3 washing steps with the IF buffer, the organoids were incubated overnight with the respective secondary antibody, diluted 1:1000 in buffer (IF-buffer with 10% BSA (w/v)). The organoids were then washed with salt-free water, followed by PBS.

To improve imaging quality, all samples were cleared with methanol: after 1 h incubation at room temperature with PBS/methanol (1/1, v/v) a second incubation with 100% methanol was performed. Both steps were carried out at room temperature under gentle agitation. As much methanol as possible was removed and 2 drops of Fluoroshield™ with DAPI were added. Microscopic slides were prepared with thick adhesive tape on both sides. The organoids were slowly transferred to the middle of the slide with a pipette. A cover slip was placed on top of the adhesive film to prevent smashing the organoids. If necessary, more Fluoroshield™ was added to encompass the entire area under the cover slip. The cover slips were left at room temperature overnight to dry for imaging the following day.

Microscopy and image processing

Light sheet microscopy was performed using a Leica DMi1 microscope. Confocal laser scanning microscopy (CLSM) was performed at the Nikon Imaging Center of Heidelberg University using a Nikon AX eclipse Ti2 with a silicon oil immersion objective (Nikon PLAN APO 40 ×/1,25 SIL λS) and the NIS Elements software (Nikon Instruments, Inc.).

Image processing was performed using ImageJ2 (as part of Fiji)[51]. Z-stacks were imaged and used for maximum intensity projections and orthogonal views. The organoid circumference was determined using the measurement function for the region of interest (ROI). Brightness and contrast adjustments were always applied linearly over the entire image. These parameters were set qualitatively for each

image. If the settings were identical for quantitative comparison, this is indicated under the respective figure.

For the quantitative analysis of cleaved caspase 3 (Cas3) staining, the proportion of the positive region in the ROI (the entire organoid area in the respective z-slice) was determined. Therefore, raw unprocessed 16-bit images were used. The respective fluorescence channel was separated and the positive proportion was determined using the threshold function in ImageJ. At equal intervals 3 z-slices within the first 50 µm of each organoid were analyzed. The results for each staining, imaging and organoid generation were normalized to the respective mean of the day 3 sample.

D-Tyr(Me)Orn and drug uptake in organoids

For all uptake experiments, organoids were generated as described and cultured until day 3. For modulation of paracellular tightness, the endothelial cell layer was compromised with multiple orthogonal approaches by pre-incubation with either 20 or 200 ng/mL of VEGF, 1 and 10 µM of adenosine in organoid medium, 5 mM EGTA, 0.5 mM EDTA solution, or a 1:10 dilution of trypsin in PBS. Incubation times are indicated on the respective graph. The control group was incubated with fresh organoid medium. After pre-incubation, the solutions were removed and 300 µM D-Tyr(Me)Orn in organoid medium supplemented with 7% human serum albumin was added. The samples were then incubated for 1 h at 37 °C under gentle agitation in the CO_2 incubator.

For drug uptakes, the respective drug was diluted in organoid medium supplemented with 7% human serum albumin to the concentrations indicated in Table 1. The incubation was performed for the time described in the graphs at 37 °C under gentle agitation in the CO_2 incubator. Time-dependent uptake was performed to determine the steady-state concentration.

After the respective incubations, the spheroid plates were placed on ice and washed five times with ice-cold PBS supplemented with 2% BSA, followed by one final wash with pure PBS. As much of the remaining liquid in the wells as possible was removed. The presence of organoids was verified under the light microscope. The organoids were lysed enzymatically by adding 20 µL of collagenase IV (3 mg/mL in PBS) to each well and incubating under agitation for 1 h at 37 °C. Then, 20 µL of trypsin solution was added, and the plate was incubated further at 37 °C for 1 h. Lysis was finalized by adding

10 µL of a 28% aqueous ammonia solution. Complete and reproducible lysis was verified under a light microscope.

For the fluorescent marker substances hypericin (2 µM) and Cy5 (300 µM), incubation was performed accordingly. After incubation, the organoids were washed and fixed for 1 h with 4% formaldehyde. The organoids were fixed on microscopic slide as described for immunofluorescence analysis with Fluoroshield™ with DAPI. Additionally, the fluorescence in the wash solutions was determined using a SpectraMax® with and without pre-incubation with 200 ng/mL VEGF for 4 h (Cy5: 630/670 nm; hypericin: 590/650 nm).

Viability and cytotoxicity assay

Organoids ($n \geq 3$) were incubated with the respective highest drug concentration for the longest used incubation time, thereby also including the evaluation of the highest DMSO concentration used. Subsequently, viability was determined using the CellTiter-Glo® 3D Cell Viability Assay as recommended by the manufacturer. The sample and reaction solution volumes were set to 50 µL. The negative control was treated with 1% Triton-X-100 and the positive control was left untreated. Background controls were performed for the medium and drug solutions. Luminescence was measured using a SpectraMax® plate reader.

Cytotoxicity was measured by adding 100 µL of the incubation supernatant to 100 µL of freshly prepared LDH assay reaction solution. Absorption was determined using a SpectraMax® plate reader at 490 nm. The positive and negative controls were reversed by definition. Normalized viability and cytotoxicity were then calculated using the formula:

$$viability/cytotoxicity\ [\%] = \frac{sample - negativ\ control}{positive\ control - negative\ control} \times 100$$

Sample preparations and analytical method for D-Tyr(Me)Orn UPLC-MS/MS quantification

D-Tyr(Me)Orn was quantified as previously reported[50]. For organoid samples, 25 µL of the internal standard (IS) D-Tyr([$^2H_3$]Me)Orn were added to approximately 50 µL of lysed organoid. After shaking the solution on a plate shaker and pipetting it up and down, 35 µL were transferred to a new 96-well microtiter plate. Calibration and QC solutions were prepared in the organoid matrix: 1,500 cells of each cell type were incubated as described for organoid lysis with 20 µL of collagenase IV, 20 µL of trypsin

solution and 10 µL of 28% aqueous ammonia. This solution was manufactured on a larger scale and stored in aliquots at -20 °C. 50 µL of this matrix were mixed with 25 µL of the IS solution and 35 µL were transferred to a new plate. The respective calibration or QC solution was then added. The remaining sample preparation and UPLC-MS/MS were identical to those published previously[50]. In brief, 25 µl of H_2O/ACN (75/25, v/v) + 0.1% FA were added to the study samples to compensate for the volume of the calibration or QC solution. Then, protein precipitation was performed with 100 µL of ACN + 0.1% FA. After shaking, the plate was centrifuged and transferred into the sample manager of the UPLC-MS/MS system. Quantification was performed on a triple quadrupole system TQ-XS (Waters, Milford, MA, USA) in positive ion mode with collision-induced dissociation using argon gas (CID) after z-electrospray ionization (ESI) and multiple reaction monitoring (MRM).

Drug quantification

All UPLC-MS/MS assays in this study were performed on triple quadrupole tandem mass spectrometers with z-ESI, stepwave optimization and CID (Waters Xevo TQ-XS and two separate TQ-S systems: TQ-S1 was coupled with a Waters Binary Solvent Manager Acquity Classic UPLC®, and TQ-S2 was coupled with UPLC I-class® and Waters Binary Solvent Manager FTN®). The systems were coupled with a Waters Acquity Column and Binary Solvent Manager. The respective analytical parameters of the MS and the chromatography for all drugs tested (Table 1), are listed in Supplement Table S1. The analytical parameters for all drugs were determined by the automatic IntelliStart software after direct infusion into the MS system.

Organoid matrix was used as a matrix for calibrator and QC samples, as described for D-Tyr(Me)Orn quantification. To 50 µL of the organoid matrix, 25 µL of the respective IS was added. After thorough mixing, 35 µL of the solution were transferred. Next, 25 µL of the respective calibration and QC solutions were added. For the study samples, each lysed organoid was mixed with 25 µL of the IS, and then 35 µL were transferred into a fresh plate. Sample preparation was then performed as summarized in Table 2. All sample preparations were performed in 96-well plates, and the transfer steps were carried out with a multichannel pipette. Centrifugation steps were performed for 10 min at 1000 × *g* after the protein precipitation. The solvent was evaporated for 15 min at 40 °C under nitrogen. The mass spectrometry and chromatography parameters for olaparib, and ulixertinib have already been published

previously[52,53], and the sample preparation and calibration range herein were adapted for the organoid matrix. The UPLC-MS/MS octreotide assay was validated for a first-in-human clinical trial[54] and herein adapted.

Calculation of intraorganoidal concentration and organoid uptake ratio

The intraorganoidal concentrations were calculated based on the approximated organoid volume after UPLC-MS/MS quantification of the respective lysis concentrations. For this purpose, the organoids were considered to be spheres with a radius of 100 µm. The concentration in the organoid before lysis was then calculated by multiplying the measured concentration in the lysis volume by the dilution factor. For all measurements, the concentration in empty but otherwise equally treated wells was determined, and the mean of that background subtracted if relevant.

The organoid uptake ratio was calculated and then correlated with the reported brain-to-plasma ratios *in vivo*. With the approximation, that:

$$\frac{intraorganoidal\ concentration}{incubation\ concentration} \approx \frac{brain\ concentration}{plasma\ concentration}$$

Therefore, at each time point after steady-state concentration was achieved, the mean intraorganoidal concentration was divided by the respective incubation concentration at that time point. These means were used as the replicates for subsequent correlation as described in the respective section. The reported *in vivo* ratios of either brain-to-plasma or CSF/unbound brain-to-free plasma concentration were used to calculate the mean with respective error. Unbound plasma concentration was determined based on the respective protein binding to calculate the unbound partition coefficient. If all values were reported with their respective standard deviation (SD), error propagation was used to calculate the error of the mean. If error values were not completely reported, the error was approximated as the standard deviation of the mean.

***In silico* BBB permeability prediction**

For the herein tested set of drugs, the respective BBB permeability predictions of four readily available *in silico* tools were determined. These were: quantitative predictions of BBBper (https://bbbper.mdu.ac.in/,[55]) and logBB-Pred (https://ssbio.cau.ac.kr/software/logbb_pred/,[56]) as well

as binary predictions by ADMETboost (https://ai-druglab.smu.edu/admet,[57,58]) and SwissADME (https://www.swissadme.ch/,[59,60]).

In vivo-in vitro and *in vivo-in silico* regression analysis

Regression analyses were performed using R version 4.3.1 (R Foundation for Statistical Calculations, Wien, Austria). For all regression analyses the untransformed correlations and the natural logarithm transformations (on one or both axes) were calculated and compared. Residuals, homoscedasticity, normal distribution and linearity were analyzed using the supporting QQ-plots, residuals vs fitted values, scale-location-diagram and Cook's distance or residuals vs index (Deming).

Because both, the organoid uptake ratios and the literature values were subject to uncertainty, a Deming-regression was performed using the Deming-package. The organoid uptake ratio replicates were used to calculate the mean and SD, to ensure equal weighting. For literature ratios x, not all replicate values were available, so the transformed standard error log(Δx) was estimated as follows:

$$\log(\Delta x) \approx \frac{\Delta x}{x}$$

For repotrectinib the CSF of only one volunteer was reported. The missing SD for the human and primate correlation was calculated using linear regression imputation based on the remaining data of the respective regression.

The 95% confidence intervals for both types of uncertainty were calculated using nonparametric bootstrapping (R=1000 replications).

For the regressions involving values without error (*in silico* tool values), weighted least squares regression were performed calculating 95% confidence intervals with build-in functions. The weights were defined as follows:

$$w = \frac{1}{\Delta y^2}$$

And for natural logarithm transformed values:

$$w_{log} = \frac{1}{(\frac{\Delta y}{y})^2} = \frac{y^2}{\Delta y^2}$$

where w (and w_{log}) represents the statistical weight assigned to each data point in the regression, y the predicted (dependent) variable, and Δy the standard deviation of y.

The regressions were evaluated using the regression coefficient R^2, which is calculated as the squared Pearson correlation coefficient. Because Deming regression does not have an associated R^2-type measure, the Pearson correlation coefficient was used as a proxy for linear association strength reported as pseudo-R^2.

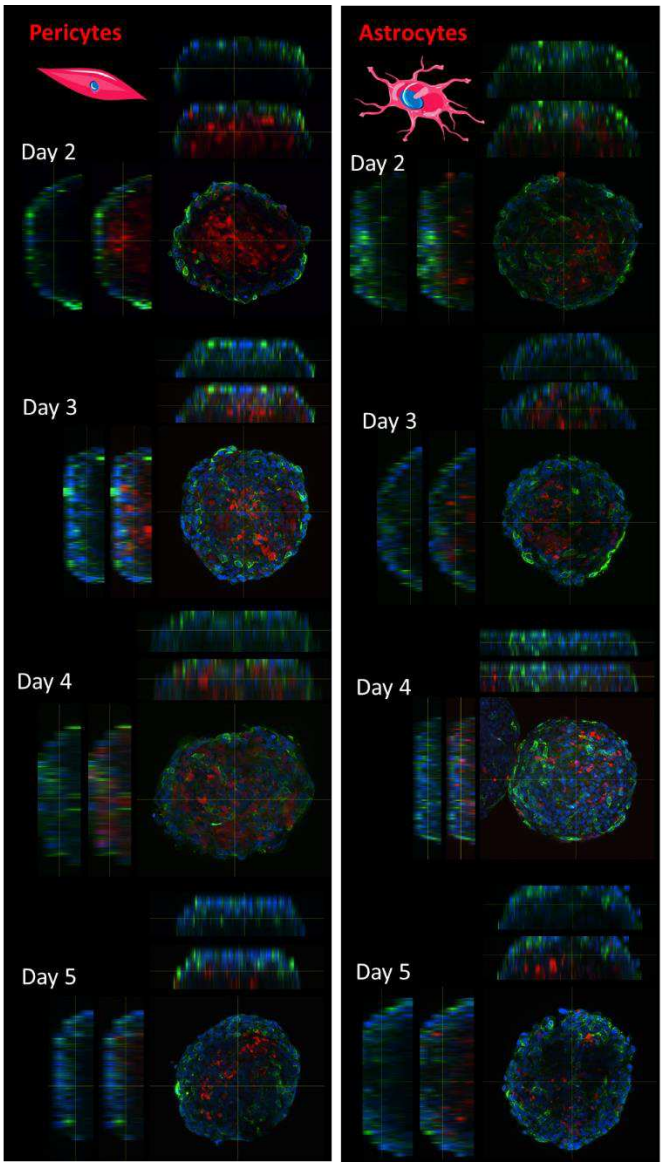
Data analysis, statistical methods and illustrations

The Waters TargetLynx V4.2 software (Waters, Milford, MA, USA) was used for quantification with UPLC-MS/MS. This was based on $1/x^2$-weighted linear regressions of the analyte response versus the respective IS response. The resulting calibration curve was used to quantify the study samples. GraphPad Prism was used for standard data visualization and statistical analysis, as indicated on the respective figures. Significances were displayed as follows: $p \leq 0.0001$ ****; $p \leq 0.001$ ***, $p \leq 0.01$ **, $p \leq 0.05$ *, p-values over 0.05 were considered not significant. ChemDraw (version 23.1.2) was used to draw the chemical structures and calculated clogP. Microsoft Excel and PowerPoint (Mountain View, CA, USA) were used for data processing and visualization respectively. Figures were created with adapted illustrations from Smart Servier (https://smart.servier.com/), licensed under CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/) and illustrations created in BioRender (Bay, C. (2026) https://BioRender.com/yaevw7j), including the graphical abstract.

Results

Evaluation of 3D BBB organoid architecture

The morphology of the cells and their overall organoid architecture were evaluated in detail over the course of the culture time. Therefore, the immunofluorescence and staining protocols were optimized to include the organoid core, with longer incubation times and methanol clearing. Although simultaneous staining with different CellTracker™ dyes would have been preferable, CellTracker™ Deep Red exhibited superior properties compared to the other tested CellTracker dyes (Green CMFDA, Violet BMQC, VivoTrack 680 near infrared), particularly in terms of even distribution throughout the cells over time. Therefore, only this dye in combination with effective CD31 staining of BCEC was used. The architecture of the organoids was imaged in depth over the course of the culture: Fig. 1 shows the organoids from days 2 to 5, with emphasis on the hCMEC/D3 coverage. Detailed images of the astrocyte and pericytes on day 3 are shown in Fig. 2A-D, remaining time points can be found in detail in Supplementary Fig. 1. The morphology of the astrocytes and pericytes was harder to depict after day 4, based on the uneven CellTracker™ staining. The majority of organoids demonstrated correct architecture, while some organoid preparations showed astrocytes on the outside. However, an endothelial layer was present beneath these outside astrocytes (see Supplementary Fig. 2). Additionally, the expression of the crucial BBB hallmarks (ZO-1 and P-gp) and the collagen IV matrix were verified (Fig. 2E).



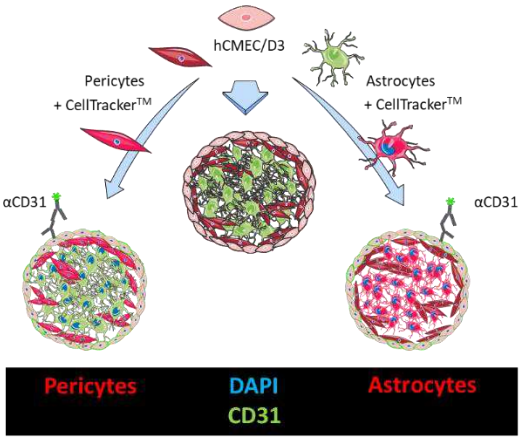


Figure 1: Representative CLSM images of the organoid architecture over culture time from day 2 to day 5 after assembly. The staining strategy is shown on the right. hCMEC/D3 cells were stained with a CD31-antibody (green). Cell nuclei are labeled with DAPI (blue). Left panel: Organoid architecture from day 2 to day 5. Pericytes or astrocytes were stained with CellTracker™ Deep red as indicated on the top. Orthogonal views of the z-stack are shown over and left side of the organoid z-slice, with DAPI and CD31 staining, as well as in combination with the CellTracker™ staining of the two different cell types. All images are representative for at least three independent organoid preparations. Scale bars were not included to maintain clarity. Illustrations were taken and adapted from Smart Servier for this figure.

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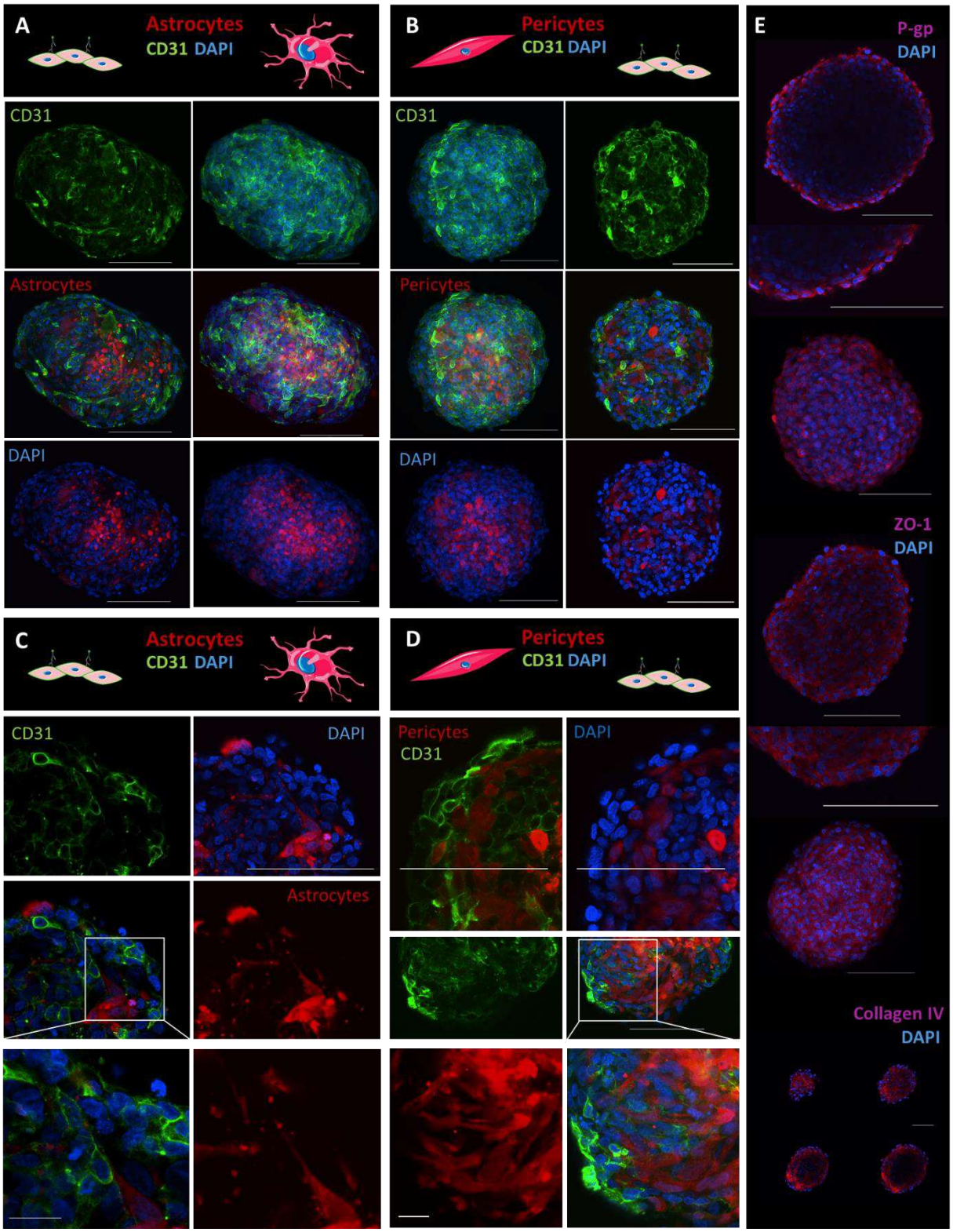


Figure 2: Representative CLSM images of the detailed organoid architecture and cell morphology on day 3. All nuclei were stained with DAPI (blue). Either astrocytes or pericytes were stained with CellTracker™ (red). hCMEC/D3 cells were labeled with anti-CD31 immunofluorescent staining (green) and BBB hallmarks P-gp, ZO-1 and Collagen IV immunolabeled in magenta. **A** Organoids with stained astrocytes (red) and hCMEC/D3 (green), on the left side z-slices with the respective channels are shown and on the right side the corresponding z-projections. **B** Organoids with stained pericytes (red) and hCMEC/D3 (green), on the right side z-slices with the respective channels are shown and on the left side the corresponding z-projections. **C** Detailed view of the astrocytes in the organoid with splitted fluorescence channels. Lowest panels show magnification of the write rectangle, to highlight astrocyte process with end in contact with BCEC. **D** Detailed view of the pericytes in the organoid with splitted fluorescence channels. Lowest panels show magnification of the write rectangle, to highlight

cell elongations. **E** Representative immunofluorescence images of organoids (from top to bottom): P-gp immunostaining z-slice, magnification of outer organoid edge and a z-projection; ZO-1 immunostaining z-slice, magnification of outer organoid edge and z-projection; collagen IV immunostaining four ascending z-slices. All scale bars = 100 µm, except in magnifications of **C** and **D** scale bar = 20 µm. All images are representative for at least three independent organoid preparations with each at least three organoid images per experiment. Cell illustrations taken and adapted from Smart Servier.

Evaluation of apoptosis during culture by Cas3 staining

Firstly, the average size (measured as circumference) was determined over time. As depicted in Fig. 3A, the organoids did not grow significantly during the first 5 days, compared to day 2 after assembly. Only on day 6, the circumference was significantly larger. The extent of apoptosis was visualized within the relevant time frame with preferred architecture from day 2 to day 4 using Cas3 immunofluorescent staining. As shown quantitatively in Fig. 3B and with representative images in Fig. 3C, the apoptosis decreases after an initial peak on the day after generation. Over time, the Cas3-positive regions appear to move closer to the surface region of the organoid. Based on these results in combination with the architecture over time, day 3 was selected as the optimal time point for experiments.

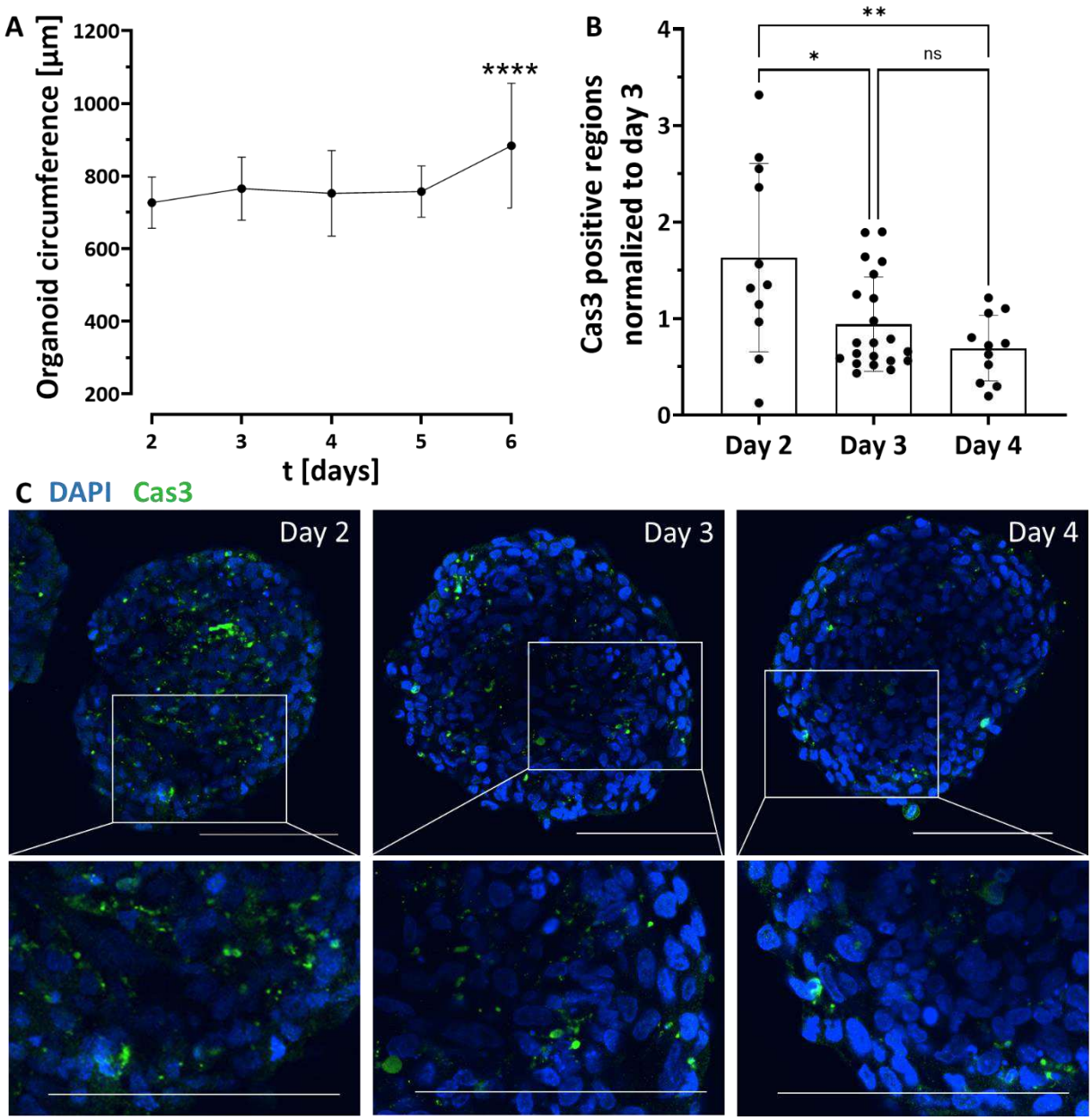


Figure 3: Evaluation of organoid size and apoptosis over time. A Organoid circumference from day 2 to day 6, shown as mean ± SD of at least 18 individual organoids from four independent experiments. Significant enlargement at day 6, one-way ANOVA with Dunnett's posthoc test compared to day 2. **B** Share of Cas3 positive region between day 2 and day 4. Depicted as mean ± SD of at least eleven individual organoids from at least three independent experiments, normalized to day 3 of each experiment. Each point represents one organoid. Statistical analysis with one-way ANOVA with Turkey's correction for multiple testing comparing all groups with each other was performed. **C** Representative CLSM images of Cas3 immunofluorescent staining. On each day an organoid is shown with z-slice and one enlargement as indicated with the white rectangle. Images were processed equal for comparison.

D-Tyr(Me)Orn demonstrates modular paracellular tightness of organoids

Our previously published analytical assay with the established paracellular marker D-Tyr(Me)Orn[50] was validated in the organoid matrix, enabling its use in analyzing the integrity of organoids (Supplement Table S2).

To modify the paracellular integrity of the organoids, the cell-to-cell connections were compromised using different modulators: VEGF, adenosine, trypsin, EGTA and EDTA. This pretreatment significantly reduced the amount of the paracellular marker inside the organoids (Fig. 4A-D) indicating an increased washout of D-Tyr(Me)Orn with reduced tightness of the organoids in our experimental setting that requires six washing steps for the required sensitivity of drug uptake quantification. These results are counterintuitive, so we investigated the washout of two small fluorescent compounds using the same settings as before, but with a detection method that has a higher dynamic range: (1) Cy5, a highly hydrophilic marker, which can only reach the inner part of the organoid paracellularly and (2) hypericin, a highly lipophilic compound which can penetrate the endothelial cells via the plasma membrane. The respective distribution of the two marker compounds was evaluated with CLSM (Fig. 4G). This revealed a cell staining with the lipophilic fluorescent hypericin, while Cy5 was mainly found in the presumed extracellular space and next to nuclei showing signs of apoptosis verifying the different routes of uptake into the organoids. Quantifying the fluorescence after different washing steps revealed a higher fluorescence of Cy5 in the wash solutions in the VEGF-pretreated group. However, this difference was only significant in the third washing step (Fig. 4E). In contrast, the washout of the lipophilic compound hypericin did not increase in the VEGF-pretreated group, but was even lower in the first washing step after VEGF incubation (Fig. 4F).

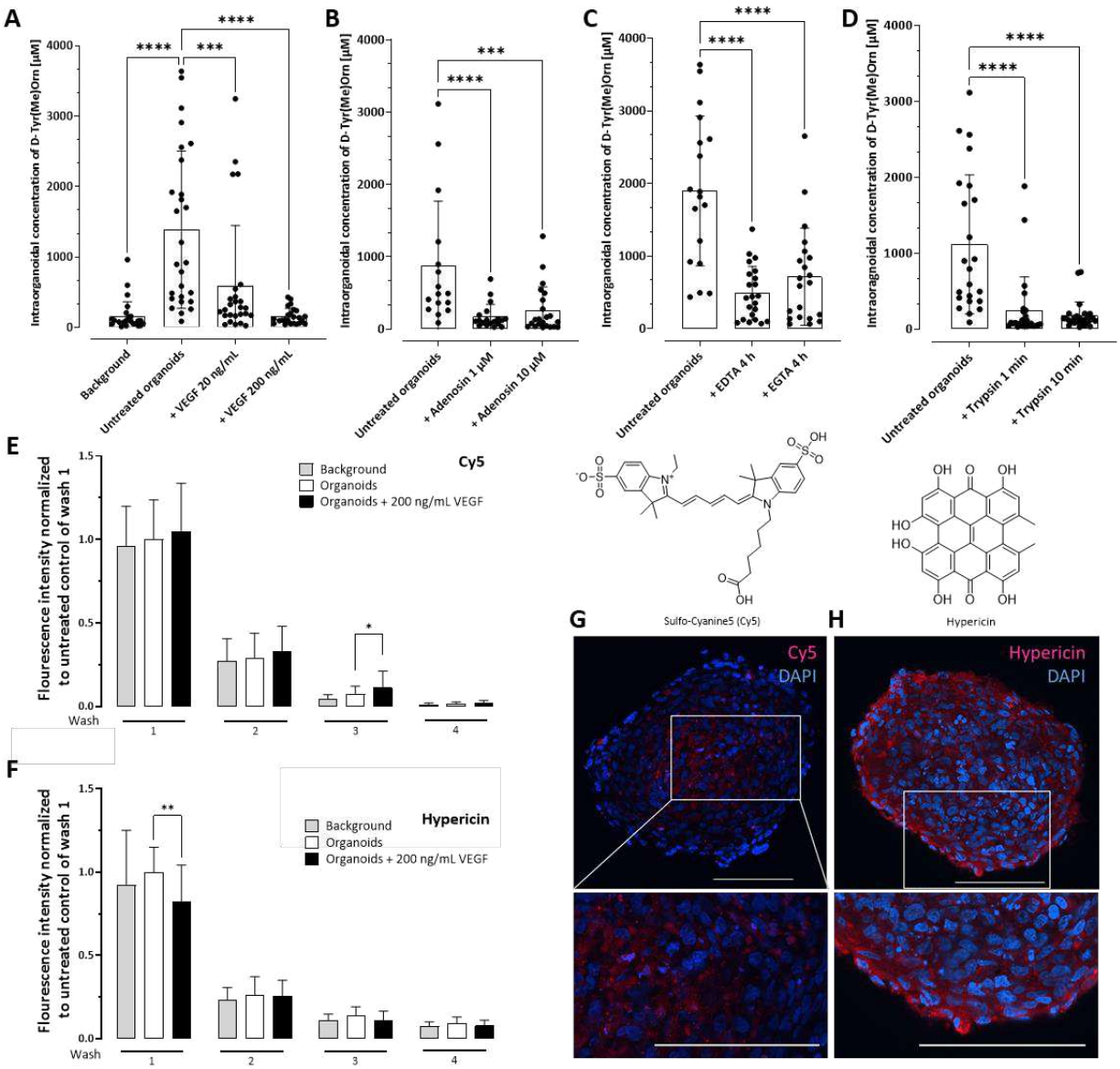


Figure 4: Evaluation of paracellular integrity of the organoids. Cell connections were compromised with the indicated pretreatment **A** 20 or 200 ng/mL VEGF for 4 h, **B** 1 and 10 µM adenosine for 1 h, **C** 5 mM EGTA or 0.5 EDTA solution for 4 h **D** trypsin solution diluted 1:10 for 1 and 10 min after the six washing steps as developed for the UPLC-MS/MS setup. All pretreatments significantly reduced the intraorganoidal concentration of D-Tyr(Me)Orn compared to untreated organoids. Data presented as mean ± SD. Probable outliers were removed with GraphPad Prism. Each point represents an individual organoid from three independent experiments with each at least four organoids. **E** Accordingly, the fluorescence of Cy5 in the wash solution for washing step 3 was significantly higher in the VEGF pretreated group, **F** while the amount was reduced for the lipophilic marker hypericin in washing step 1. **G** Cy 5 and **H** hypericin were also visualized under the fluorescent microscope. Respective structures are depicted on top. These two markers showed a distinct distribution in the organoids (both red): hypericin stained the cells and Cy5 was contained to the extracellular space. Representative z-slices of organoids of three independent experiments, and respective detailed presentation. Cell nuclei stained with DAPI (blue). Scale bar = 100 µm. All statistical analysis were performed with one-way ANOVA with Dunnett's post-hoc test.

Experimental setup for UPLC-MS/MS quantification of drug uptake in organoids

Quantification on single organoid level using UPLC-MS/MS was challenging due to the difficulty of measuring low concentrations in a small number of cells. Transfer steps should be avoided due to the potential for disruption of the barrier. This required an experimental setup with easy access for washing, feasible for automation. Based on the use of spheroid plates, the organoid generation and whole drug exposure until lysis could be performed in one format (Fig. 5). Excessive washing is required due to the tiny intraorganoid volume available for drug uptake in combination with expected low permeation through the hCMEC/D3 layer and the high concentration of substance in the incubation solutions. Additionally, to avoid losing organoids when the solution is removed after incubation and during each washing step, a residual volume of 10 µL must be left in the wells. Six washing steps ensured the required reduction of the background caused by remaining volume of substance solution in the wells (~ 10 µL remaining, wash with 150 µL) resulting in the possibility to detect intra-organoid concentrations of 1% of the incubation solution. This protocol resulted in an acceptable loss of ~ 5% of the organoids per plate (39 plates with a total of 2,682 organoids, 154 organoids were lost; with a mean loss of 5.5 ± 4.0% per plate).

A particular challenge for analysis was the organoids resistance to lysis due to the strong cellular and extracellular connections. The organoids were resistant to basic and acidic lysis, organic solvents, vigorous mixing, and ultrasound, in accordance with collagen expression (Fig. 2E). The goal was to completely disassemble the organoids and completely lyse cells for a reproducible lysis protocol and accurate quantification. The final protocol involved enzymatic digestion (1 h collagenase IV and 1 h trypsin/EDTA solution) followed by ammonia lysis. This achieved complete dissolution of the organoids (Supplementary Fig. S3).

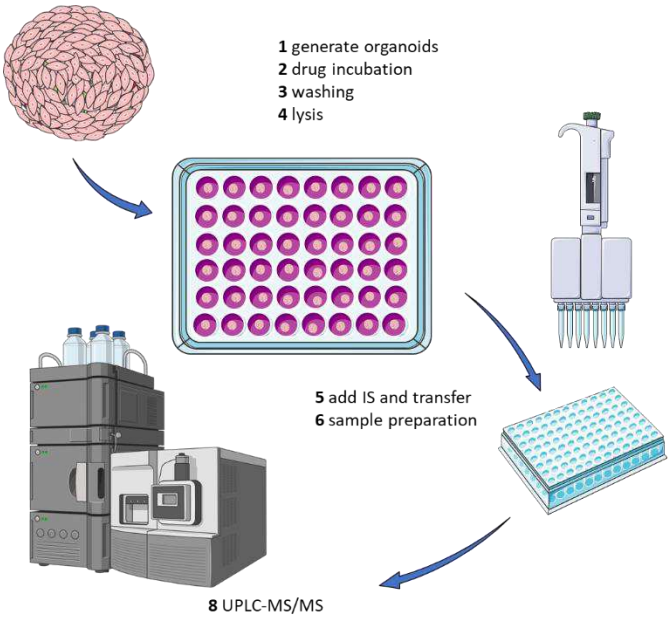


Figure 5: Experimental setup for UPLC-MS/MS assay in 3D BBB organoids. Schematic overview of the organoid assay for UPLC-MS/MS quantification. Organoids are generated directly in the coated spheroid plates and incubated with the respective drug on day 3. After incubation, organoids are washed 6 times and digested with collagenase IV and trypsin. After ammonia lysis, the IS is added. Then, the samples are transferred to a new plate and subjected to the respective sample preparation and UPLC-MS/MS quantification. Created with adapted illustrations from Biorender and Smart Servier.

Correlation of organoid drug uptake with *in vivo* BBB permeability data for evaluation of the translational relevance of the 3D BBB organoid model

All drug quantifications were based on verified calibration curves using QC samples in the organoid matrix (see Supplementary Table 3 for representative QC results). Additionally, the stability of the drugs during the organoid lysis was confirmed. The concentration of the drugs in the organoid was then determined concentration- and time-depended, as depicted in Fig. 6, A. A time-dependent assessment is necessary to identify the relevant concentration equilibrium for accurate organoid uptake ratio calculation. Concentration-dependent assessment is required to identify nonlinear effects, such as transport saturation. All uptakes were performed in three independent experiments. Not all incubations and time points resulted in measurable organoid concentrations. Values were accepted for downstream analysis if at least seven individual organoids from two independent organoid preparations were quantifiable. For octreotide, the concentrations in the organoids equaled the background concentrations and were therefore interpreted as negligible organoid uptake. This zero-point was not used for correlation and only included in the following plots for visualization. Overall, organoid concentration was linear to increasing incubation concentration. Saturation of uptake was achieved for all drugs within the observed time frame. Steady state was presumed, when there was no significant concentration increase between the time points (see Supplementary Table S4). After reaching steady state, the ratio of the mean organoid uptake concentration to the respective incubation concentration was calculated for

each time point (= organoid uptake ratio). These ratios, each based on at least ten individual organoids, were used as replicates for the correlation with reported *in vivo* brain-to-plasma ratios (Fig. 6B). The *in vivo* data can be seen in detail in Table 3. Either the brain tissue-to-total plasma ratio or the unbound brain- or CSF- to-unbound plasma ratio was used (please refer to the discussion for a detailed description of the literature data). Concentrations too close to the measured background were excluded to avoid artifacts. Reduction of viability and cytotoxic effects were evaluated for all drugs used (Fig. 6C). The quality of the correlation was determined using Deming regression (Fig. 6D-F). A comparison of the mammalian correlations performed with and without transformation can be seen in Supplementary Fig. S4. Based on normality diagnostic plots, a transformation using the natural logarithm of both axes was selected for the final correlation (see Supplementary Fig. S5). This resulted in a pseudo R^2 of 0.64 for the Deming regression between organoid uptake ratios and the complete mammalian data set for *in vivo* brain-to-plasma ratios (Fig. 6, D). Sub-analysis with the reported primate data provided a pseudo R^2 of 0.81 (Figure 6, E), and regression solely based on human data resulted in a pseudo R^2 of 0.82 (Figure 6, F).

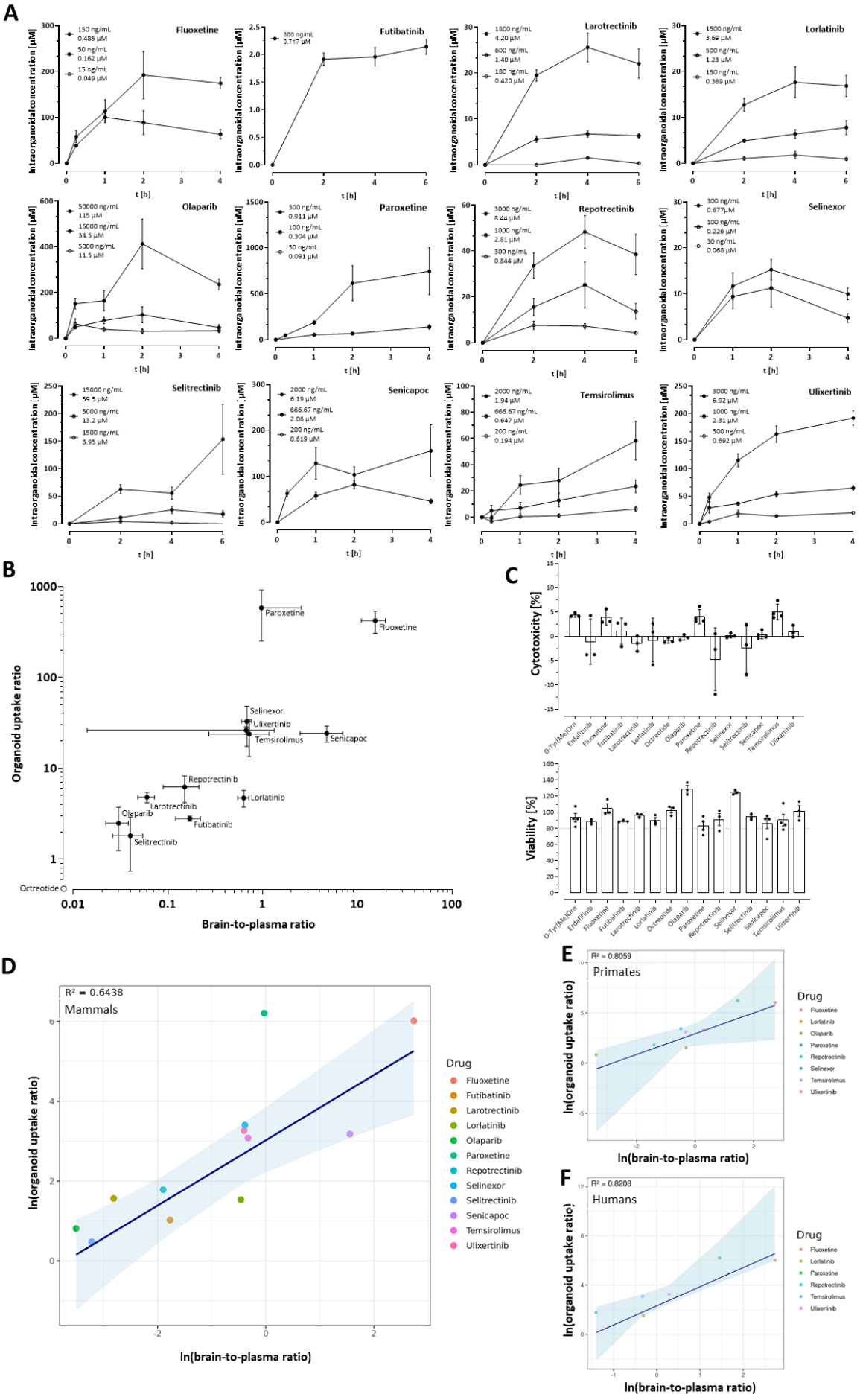

A
Fluoxetine
150 ng/mL 0.485 µM
50 ng/mL 0.162 µM
15 ng/mL 0.049 µM
Futibatinib
300 ng/mL 0.717 µM
Larotrectinib
1800 ng/mL 4.20 µM
600 ng/mL 1.40 µM
180 ng/mL 0.420 µM
Lorlatinib
1500 ng/mL 3.69 µM
500 ng/mL 1.23 µM
150 ng/mL 0.369 µM
Olaparib
50000 ng/mL 115 µM
15000 ng/mL 34.5 µM
5000 ng/mL 11.5 µM
Paroxetine
300 ng/mL 0.911 µM
100 ng/mL 0.304 µM
30 ng/mL 0.091 µM
Repotrectinib
3000 ng/mL 8.44 µM
1000 ng/mL 2.81 µM
300 ng/mL 0.844 µM
Selinexor
300 ng/mL 0.677µM
100 ng/mL 0.226 µM
30 ng/mL 0.068 µM
Selitrectinib
15000 ng/mL 39.5 µM
5000 ng/mL 13.2 µM
1500 ng/mL 3.95 µM
Senicapoc
2000 ng/mL 6.19 µM
666.67 ng/mL 2.06 µM
200 ng/mL 0.619 µM
Temsirolimus
2000 ng/mL 1.94 µM
666.67 ng/mL 0.647 µM
200 ng/mL 0.194 µM
Ulixertinib
3000 ng/mL 6.92 µM
1000 ng/mL 2.31 µM
300 ng/mL 0.692 µM
Intraorganoidalconcentration [µM]
t [h]
B
Organoid uptake ratio
Brain-to-plasma ratio
Paroxetine
Fluoxetine
Selinexor
Ulixertinib
Temsirolimus
Senicapoc
Repotrectinib
Lorlatinib
Larotrectinib
Olaparib
Futibatinib
Selitrectinib
Octreotide
C
Cytotoxicity [%]
Viability [%]
D-Tyr(Me)Orn
Erdafitinib
Fluoxetine
Futibatinib
Larotrectinib
Lorlatinib
Octreotide
Olaparib
Paroxetine
Repotrectinib
Selinexor
Selitrectinib
Senicapoc
Temsirolimus
Ulixertinib
D
R² = 0.6438
Mammals
ln(organoid uptake ratio)
ln(brain-to-plasma ratio)
Drug
Fluoxetine
Futibatinib
Larotrectinib
Lorlatinib
Olaparib
Paroxetine
Repotrectinib
Selinexor
Selitrectinib
Senicapoc
Temsirolimus
Ulixertinib
E
R² = 0.8059
Primates
F
R² = 0.8208
Humans

Figure 6: A Concentration-time profile of the quantified drugs in the organoid. These were used to determine the steady state time and to calculate the respective organoid uptake ratios for the correlation. Data depicted as mean ± SEM of at least seven organoids from at least two independent organoid preparations**. B** Correlation of organoid uptake ratios with reported *in vivo* brain-to-plasma ratios. The quantified drug concentrations in the organoid were used to calculate the ratio to the incubation concentration (organoid uptake ratio). These are depicted against the reported *in vivo* brain-to-plasma ratios (Table 3). Data is shown as mean ± error calculated as described in the method section. Based on logarithmic axis, the zero data point of octreotide cannot be depicted and was inserted afterwards for conceptual visualization. **C** Viability (ATP) and Cytotoxicity (LDH) after incubation with the highest used concentration for the longest observed time point. Mean ± SD of at least three replicates. Each point represents an individual organoid. **D** Deming regression of the measured organoid uptake ratios with reported mammalian *in vivo* brain-to-plasma ratios and **E** with only the reported primate *in vivo* data and **F** only the human *in vivo* data. The correlations were performed using natural logarithm-transformed data. Respective pseudo R^2 and the 95% confidence interval are depicted. The Deming regression calculated the insecurities of both axes. Data points represent the mean of the ratios of the individual drugs, the error values were transformed as described in the method section.

Regression of the *in vivo* BBB permeability data set with results from *in silico* BBB prediction tools reveals absent linear correlation

For the selected drug set, the BBB permeability predictions of four *in silico* web tools were determined and correlated with the researched *in vivo* data set. Two of the tools provided quantitative results: the BBB Score [%] of ADMETboost and logBB-values of the logBB-Pred tool (Fig. 7A and B). As performed for the correlation with the organoid uptake data, natural logarithm transformations were performed, and the best hits were presented (Fig. 7C and D, comparisons of direct and transformed correlations are in Supplementary Fig. 4, transformed diagnostic plots are in Supplementary Fig. S5). Because only the *in vivo* data had a reported error, a weighted regression was performed. However, no linear correlation was seen for the selected drug set with either tool (R^2 of 0.06 and 0.13). The binary results (BBB permeable/non-permeable) of SwissADME and BBBper also did not result in a meaningful pattern (Fig. 7E and F).

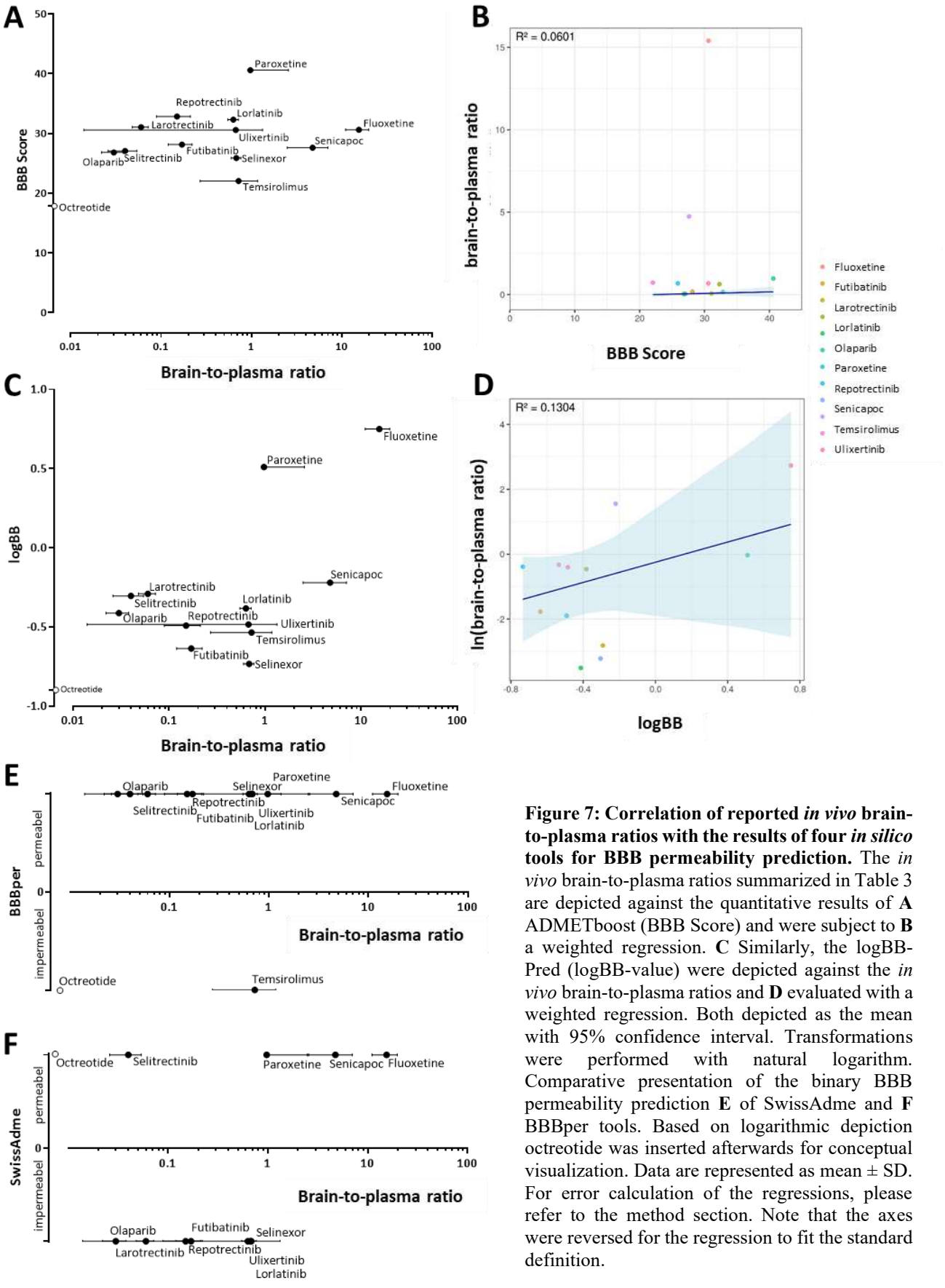


Figure 7: Correlation of reported *in vivo* brain-to-plasma ratios with the results of four *in silico* tools for BBB permeability prediction. The *in vivo* brain-to-plasma ratios summarized in Table 3 are depicted against the quantitative results of **A** ADMETboost (BBB Score) and were subject to **B** a weighted regression. **C** Similarly, the logBB-Pred (logBB-value) were depicted against the *in vivo* brain-to-plasma ratios and **D** evaluated with a weighted regression. Both depicted as the mean with 95% confidence interval. Transformations were performed with natural logarithm. Comparative presentation of the binary BBB permeability prediction **E** of SwissAdme and **F** BBBper tools. Based on logarithmic depiction octreotide was inserted afterwards for conceptual visualization. Data are represented as mean ± SD. For error calculation of the regressions, please refer to the method section. Note that the axes were reversed for the regression to fit the standard definition.

Discussion

Predicting human BBB permeability is crucial for assessing the suitability of drug candidates in addition to identifying candidates with limited permeability for avoiding CNS side effects in malignancies not associated with the CNS. Unfortunately, determining BBB permeability is challenging even in the clinical settings, due to the restricted access to human CNS samples. Animal models show partly large discrepancies especially for P-gp substrates[5-10]. Although *in vitro* models lack pharmacokinetic recapitulation, they can be composed of human cells only. Complex models based on different cell types of the neurovascular unite have been shown to be relevant in pathophysiological modeling[61,62]. In general, accessibility to the basolateral compartment, and therefore to the sample behind the BBB, decreases as complexity increases[62-64]. For example, in complex BBB-on-a-chip-models, the readout is restricted to fluorescent substances[63] or requires the application of pressure[65]. However, accessibility for quantification is a main quality criterion for pharmacological analysis. While larger constructs for delivery strategies can be easily labeled, dependency on fluorescence hinders the evaluation of small molecules, because fluorescent tags severely alter their physicochemical properties.

The 3D BBB organoid model is accessible for UPLC-MS/MS analysis

Previous readouts in the 3D BBB organoid model used herein were mainly based on imaging analysis with fluorescence for marker substances, targeting constructs or 2D MS technique like MALDI for exemplary uptake evaluation of two drugs[19,20,48,49]. However, not all molecules of interest can be observed with fluorescence read-out and MALDI has currently limited resolution (30-50 µm[19,49]), is not trivial, requires sample slicing and not all analytes ionize sufficiently. In contrast, 1D MS such as UPLC-MS/MS, is the gold standard for clinical drug quantification.

To our knowledge a validated assay for the quantitative analysis with simple and higher-throughput-MS has been lacking. The only previous 1D MS assay for BBB organoids was a binary evaluation as verification for an *in silico* tool without relation to the respective limit of quantification or detection[66]. Due to the absence of quantitative drug uptake measurements in such organoids we established an easy, reproducible and adaptable framework for quantifying a wide range of drugs in 3D BBB organoids. We

investigated and identified optimal organoid culture and handling conditions. Using commercially available plates, the organoid generation is faster, the risk of contamination reduced, and the plate precoating increases reproducibility. These plates allow for an easy medium change with minimal organoid loss and eliminate the need for transfer steps. This ensures compatibility with automation, when either a tilting platform or angled pipetting is available. The major bottleneck of this high-throughput assay is the LC-MS/MS quantification method development with sufficient (very low) limits of quantification. However, most small molecules (and peptide) quantifications are accessible by standard sample processing techniques like protein precipitation using organic solvents and reverse-phase chromatography, as was performed for most of the drugs in this study. Exceptions are the liquid-liquid extraction for fluoxetine and the HILIC separation for D-Tyr(Me)Orn, also delivering sufficiently low quantification limits. The developed options provide a blue print for preparing samples for a wide range of analytes.

The investigation of two fluorescent model substances (Cy5 and hypericin) verified that these drugs are not only attached to the outer cell layer, but indeed inside the organoid structure. These substances also demonstrated a strong difference between lipophilic and hydrophilic distribution within the organoids. Of note, the whole organoid lysis results in the quantification of drugs in the organoids, as well as adsorbed to and in the BCEC. However, the same is true for tissue quantification *in vivo*. Drug-specific variations can occur, especially in low-permeable substances, depending on the effects of perfusion and washing, which could potentially result in *in vitro-in vivo* artifacts.

The 3D BBB organoid model shows favorable architecture and viability on day 3 after generation

The organoids were verified to be comparable to those in previous publication after the described culture adaptions like using an orbital shaker, as well as the ready-to-use pre-coated plates. This was performed with CLSM with respect to architecture and crucial BBB hallmark expression. While previous investigations mainly focused on the overall architecture, we observed the specific morphology of astrocytes and pericytes in detail and over time. We therefore optimized the staining protocols for in detail imaging, including the selection of the most suitable dye and addition of sample clearing.

Due to the inverse and spherical architecture of the organoids, they cannot develop true BBB structure, which requires pericytes to wind helically around the vessel[67,68] and the coverage with astrocyte end feet

620 over the other two cell types[69,70]. However, the presence of elongations of both cell types demonstrates
621 that they are viable cells with a morphology that resembles *in vivo* cells and that they both interact with
622 the BCEC. Organoid models do not exhibit the physiological architecture of the microvasculature,
623 unlike self-vascularizing models in microfluidic systems[63,65]. Nonetheless, compared to these models,
624 the organoids used here show a higher accessibility to the basolateral compartment by whole organoid
625 lysis, while more closely recapitulating free cell-type interaction than Transwell® models or microfluidic
626 systems without vascularization. A meaningful future comparison would be a direct performance
627 comparison of organoids with both kinds of microfluidic models – simple BCEC layers with other cell
628 types for example in matrix gel[71] and self-vascularized structures[65].
629 BCEC are the core of each BBB model and generally benefit from additional differentiation time in the
630 final BBB model setup[63,72]. The aim is to achieve a tight monolayer. The outer surface of the organoids
631 appeared to be smoother on day 3, possibly indicating closer cell contact. After day 4 a tendency for
632 more CD31+ cells on the cell layer was identified, possibly representing the formation of a multilayer
633 to some extent. In general, hCMEC/D3 exhibits contact inhibition in 2D monoculture[73], and previous
634 publications on organoids did not observe an increase in overall organoid size[20]. However, we observed
635 an increase in organoid circumference, which was most likely due to an increase in the number of
636 pericytes over time. Because all cell types exhibited positive Ki67 staining for proliferation[49], a complete
637 contact inhibition of the hCMEC/D3 in the organoids could not be assumed. Therefore, any sign of a
638 multilayer hCMEC/D3 was closely observed, although this was hampered by the strong CD31 signaling.
639 Additionally, viability was assessed by evaluating the amount of apoptosis. The main peak on day 2 and
640 the subsequent decrease can most likely be attributed to the stress of generating the organoids, which
641 includes detaching the adherent cells. Cas3 staining was sufficient for our general evaluation, however,
642 we want to note that Bell and coworkers have developed a precise and validated toxicity screening based
643 on hematoxylin staining for these organoids[49]. Importantly, not all standard *in vitro* assays for viability
644 or cytotoxicity assessment we tested were sensitive enough for single organoids, highlighting the need
645 for evaluation in the used *in vitro* model and inclusion of negative and positive controls.
646 Eventually, in combination with the evaluation of the architecture and the hCMEC/D3 monolayer, day
647 3 was identified as the optimal time point for analyses, because it offered the best balance between the

correct cell morphology, complete and smooth endothelial layer, constant size, apoptosis and existence of a single endothelial layer.

The performed culture adaption had no impact on the previously reported correct expression patterns of ZO-1 and P-gp, which is important due to the significant impact of TJ and efflux transporters on BBB permeability. However, the mere expression of a TJ marker is not a sufficient confirmation of functionality, because TJ require correct localization and architecture[74,75]. ZO-1 expression was also observed in the cytoplasm, as is often the case with hCMEC/D3 while pure expression at the cell corners would be more representative of physiological conditions[20,76,77]. Final conclusions about barrier properties can only be drawn from a functional evaluation.

The 3D BBB organoid model shows functional barrier properties

In previous studies, TJ modulation with VEGF or histamine in 3D BBB organoids resulted in a higher intraorganoidal signal of fluorescently tagged, high-molecular-weight molecules[20,61,78]. There was no relevant uptake of these paracellular markers in intact organoids, nor was there any size-dependency observed between 4 to 70 kDa[48]. Considering the highly restrictive nature of the BBB as well as the small size of average drugs, macromolecular substances are not suitable to evaluate subtle but relevant BBB impairment. However, for smaller markers, the literature is contradicting: the organoids were shown to be impermeable to larger molecules without modulation of the barrier, but free fluorescein isothiocyanate (FITC) was able to diffuse into the organoids, without significant difference compared to those organoids without BCEC[48]. Recent results also showed a basal uptake of Lucifer Yellow in intact organoids, which in contrast was increased upon treatment with TNF-α and INF-γ[79]. Similarly, difference in the incubation solution concentration of fluorescein was shown between different organoids with iPS-derived endothelial cells[80]. Similar to FITC and Lucifer Yellow, we observed low basal uptake of Cy5 and D-Tyr(Me)Orn. The latter is a previously developed dipeptide paracellular marker that can be measured with high sensitivity using UPLC-MS/MS and is highly discriminative for TJ status due to its small size[50]. Interestingly, we did not observe an increase in the intraorganoidal concentration of the marker substance when we modulated the cell connections by disruption of the calcium-dependent junctions with EGTA/EDTA, modulating the TJ with adenosine and VEGF, or

degrading proteins with trypsin. In contrast, these treatments in our assay resulted in decreased intraorganoidal D-Tyr(Me)Orn. We hypothesize that the discrepancy's for small markers may be due to wash-out phenomenon's: The small marker D-Tyr(Me)Orn can be washed out more efficient in the compromised organoids masking or even reversing the uptake into the organoids during incubation. For Cy5 – the fluorescent pendant to D-Tyr(Me)Orn– this results in an detectable increased amount of marker in the washing solutions. However, small differences are hardly detectable due to the minimal amount of substance in the organoids compared to that in the remaining uptake solution before washing. Therefore, differences would only be visible after the remaining uptake solution is sufficiently diluted by washing steps. This is in line with what we observed for the fluorescent marker Cy5 with a higher amount detected in the washing solutions after VEGF treatment, that only is significant in step 3.

Interestingly, the hypericin washing solution exhibited the opposite effect. This may be due to a higher hypericin amount in the compromised organoids that is resistant to wash-out due to the high lipophilicity.

The larger marker substances, which only enter after cell connections are modulated, are more than 200 times larger, and therefore may show completely different behavior during washout. In addition, it is worth mentioning that other protocols are restricted to three washing steps[20,48,61], if washing steps are specified at all.

All of the assays developed herein allow for the simultaneous quantification of two analytes – one intended for the drug and one for the paracellular marker – at the single organoid level. However, D-Tyr(Me)Orn cannot be used as a threshold for organoid integrity. While D-Tyr(Me)Orn was able to identify significant effects based on the organoid integrity in the mean, the high biological variance in the untreated organoids hindered the discrimination power of the marker as a threshold on single organoid level. The basal uptake of small hydrophilic molecules (D-Tyr(Me)Orn, FITC and Cy5) must be considered, as it limits the use of small paracellular biomarkers for integrity control in such models.

The fact that hCMEC/D3 did not show a difference in permeability after EGTA treatment in the 2D Transwell® assay[50] and after VEGF treatment (see supplemental Figure S6), but caused reduced D-Tyr(Me)Orn concentration in the organoid model indicates a more highly developed BCEC phenotype in the hCMEC/D3 layer in the organoid model. This is in accordance with the observable higher

expression of P-gp and ZO-1 in the 3D organoid model compared to 2D models[20]. The use of BCEC with higher *in vitro* barrier properties, including the fast-growing field of iPS derived cells could possibly further improve the integrity of 3D organoid models[80].

Drug uptake in the 3D BBB organoids correlates with in vivo *permeability values*

As the updated classification criteria for brain tumors place greater emphasis on molecular and genetic alterations, personalized and targeted therapies are becoming the focus of clinical development[81,82]. One example is the 2026 approval of the targeted therapy tovorafenib[83]. In light of the latest research findings linking neuronal activity and Ca^{2+} signaling to glioblastoma growth[84,85], repurposing of drugs is another up-to-date approach[86-88]. Based on these developments, we selected a set of drugs with different BBB permeabilities, including P-gp and BCRP substrates, as well as different lipophilicity values and sizes (see Table 4), in order to test the translational relevance of the organoid model.

Although quantification of drug uptake is the most meaningful pharmacological evaluation of a BBB model, it has not been previously performed in 3D BBB organoids. For evaluating the translational significance, we correlated the organoid uptake ratios on a single organoid level in equilibrium with the *in vivo* BBB permeability values of the 12 drugs with measurable concentrations and reported brain-to-plasma ratios (Table 3). We included all found mammalian brain-to-plasma ratios (exceptions listed in the limitations), to generate a mean and achieve a representative *in vivo* permeability data set, resulting in the high standard deviation seen for some drugs. The correlation of the organoid uptake ratios with the reported *in vivo* values revealed a strong correlation in the Deming regression accounting for uncertainties in both variables. This results in a qualified NAM, indicating its suitability for predictive BBB permeability ranking.

The sub-analysis with pure primate and human data resulted in even higher regression coefficients. Although a direct comparison between these regression analyses is not possible, based on the deviating number of drugs with available *in vivo* data, a tendency of this fully human organoid model to better correlate with human data can be observed.

Selecting the transformation with the natural logarithm allowed for a proportional consideration of values across multiple orders of magnitude. Otherwise, high values would have a disproportionate

731 influence on the correlations (correction for scale imbalance). This transformation also corrects for a
732 higher standard deviation, which often increases with the magnitude of the measured values (correction
733 for heteroscedasticity). These corrections are clearly visible in the quantile-quantile plot (QQ) and
734 residual plots that fulfill the criteria for linearity, normal distribution, homoscedasticity and equal
735 weighting. With this regression, it becomes possible to perform predictions for drugs quantified in the
736 organoid, despite that the BBB organoid model can´t fully represent physiological states of the BBB due
737 to the lack of dynamic blood flow, as well as an inability to recapitulate the complex architecture, from
738 which the unique features of this barrier arise.
739 The drug quantifications showed a high interorganoidal variance, at least partly due to the already high
740 size variation between the organoids of approximately 10% adding to the expected biological variation
741 and organoid formation. This cannot be corrected by the standardized volume approximation, but in part
742 be counterbalanced by the possibility to perform more replicates based on the measurement on single
743 organoids. Previous publications often considered organoids as biological replicates[20,49,61]. Based on the
744 high variance observed here, multiple organoids from at least two independent preparations were
745 regarded reliable for drug quantification. Additionally, to reduce the influence of interorganoidal
746 variance, the mean of single time and concentration points in steady state was used for correlation
747 analysis.
748 Selecting physiologically relevant concentrations resulted partly in uptake amounts below the
749 quantification limit. Additionally, low concentrations within the organoid may be obscured by the
750 background (e.g., uptake with 180 ng/mL larotrectinib results in a background of ~ 70% of signal;
751 1,800 ng/mL in a background of ~ 10% of signal). Increasing the number of washing steps or the time
752 can increase the probability of washing artifacts and diffusion. Six washing steps were selected as a
753 trade-off between reducing background and washout time. All drugs except octreotide were measurable
754 above the background level, consistent with the negligible brain penetration of this octapeptide,
755 highlighting the ability of this NAM to model low/no brain permeability.
756 In contrast to the organoid *in vitro* model, the *in silico* models used herein did not show a correlation
757 with the *in vivo* data. However, at least two of the models (chemical descriptor-based) are among the
758 top-ranked current tools[89]. The quality of all trained models depends on the quality of the training

dataset. As discussed in the limitations, the BBB permeability data is of poor quality, resulting in lower information content. Larger data sets are often binary, or use thresholds for combination with quantitative data and are biased toward permeable drugs with lesser entries of non-permeable substances. This strategy depends heavily on the selected threshold and naturally neglects relevant drugs with low permeability and low effective concentration. This makes the training of quantitative models extremely difficult[57,89]. Additionally, they often incorporate the physiochemical properties, which regularly fail to truly determine the BBB permeability[2].

Limitations:

One limitation of our model is the variance in the regression which indicates uncertainty in these predictions, reflected in the confidence intervals and based on the interorganoidal variance.

A general limitation in BBB research is the current data availability and variation in acquisition. Brain-to-plasma ratios are not standardized and can differ in regard to:

1) Method of sampling. Typically, they are measured by quantifying the brain tissue concentration and the total plasma concentration (B/P or as partition coefficient $K_{p,brain}$). For substances with high platelet incorporation, determination in whole blood is more appropriate (see e.g., temsirolimus). Since only the unbound fraction of a drug can reach the target, the unbound fraction ratio (B_u/P_u or unbound partition coefficient $K_{p,uu,brain}$) was proposed as a more insightful parameter[90]. Quantifying unbound brain concentration or the extracellular fraction is not trivial. It can also be determined in different ways, either by technically challenging microdialysis[91,92] or by calculation with the beforehand identified tissue binding percentage. Although not always identical, the concentration in the CSF is used as an approximation of free brain concentration[90,91,93,94].

2) Time of sampling. Partition coefficients are determined either in steady state or after a single dose with the AUC. Unfortunately, these ratios are not always established in a time-independent manner: quantification of the B/P ratio after a single dose on a single time point depends highly on drug-specific pharmacokinetics and, therefore, on the selected time point.

3) Specie and pathophysiology. Obviously, brain-to-plasma ratios are more comparable when evaluated in healthy subjects. However, the ratios are often determined in the relevant disease model, which

786 frequently affects the BBB. Most human data are collected from patients included in clinical studies, as
787 seen here with the CSF samples for repotrectinib and lorlatinib. Therefore, and based on the smaller
788 assumed effect on CSF concentrations from a partly opened BBB, we included these data for our
789 correlation analysis. Data derived from tumor tissue was only included it if it was mixed with total brain
790 tissue or if it was shown to have no differences compared to healthy brain tissue. The exception was
791 temsirolimus, for which tumor tissue data were the only available tissue concentrations. For olaparib,
792 mice with tumors showed comparable results to healthy macaques[95]. The authors presumed this amount
793 to be negligible with no true brain penetration, consistent with quantifications in rats[96]. In contrast,
794 human tumor tissue showed a high concentration of olaparib with large variability, probably due to
795 BTB[96].
796 Ulixertinib exhibited low brain concentrations in healthy and tumor tissue of mice, resulting in a brain-
797 to-plasma concentration of 0.0176[53]. The first results of a clinical trial, presented in a poster, show higher
798 brain concentrations in tumor patients. The CSF-to-unbound plasma concentration was 1.33 with high
799 discrepancies in the tumor's bound ratios (enhancing region: 0.5; non-enhancing region: 0.2). However,
800 these results are still much larger than those observed in rodents[97]. This discrepancy may be due to
801 species differences often observed for P-gp substrates[5-10].
802 4) Inclusion of relevant metabolism. While we did not observe relevant generation of the active
803 metabolites of fluoxetine and temsirolimus in the organoid model, the amount of norfluoxetine is
804 included in the reported *in vivo* brain permeability. We therefore used these sums as approximations for
805 fluoxetine alone.
806 Ultimately, all these deviations may result in discrepancies in the reported brain-to-plasma ratios for a
807 drug. Improved *in vivo* data would benefit translational correlations for performance evaluation of BBB
808 models and training of *in silico* prediction tools.

809 **Conclusion**

810 This study established and validated the first UPLC-MS/MS assay for the reproducible quantification
811 of drugs in single 3D BBB organoids. This strategy generated the first pharmacological quantitative
812 evaluation of *in vitro*-to-*in vivo* translational relevance for drug BBB permeability in an organoid model

and qualified this strategy as a NAM for prediction and ranking of the BBB permeability of drugs *in vivo*. This NAM was demonstrated to be superior over state-of-the-art *in silico* prediction models and outperformed reported 2D BBB models. The reliable setup allows for future automation to implement this NAM into routine screening of drug candidates for CNS malignancies or to select candidates with low BBB permeability to avoid CNS side effects in toxicological characterization.

Supplementary information:

Supplement Table S1: Mass spectrometry and chromatography parameters for analyzed drugs.

Supplementary Figure S1: Representative CLSM images over culture time from day 2 to day 5 after assembly.

Supplementary Figure S2: Representative CLSM images of astrocytes located outside the organoid cover in some organoid preparations.

Supplementary Table S2: Validation results for D-Tyr(Me)Orn in organoid matrix.

Supplementary Figure S3: Representative light microscopy images of organoid lysis.

Supplementary Figure S4: Comparisons of direct and logarithm transformed correlations.

Supplementary Figure S5: Diagnostic plots for performed correlations with and without logarithmic transformation.

Supplementary Table S3: Representative quality control results for UPLC-MS/MS drug assays.

Supplementary Table S4: Statistical analysis of drug uptake in organoids over time.

Supplementary Figure S6: Clearance volume of Transwell® experiments of D-Tyr(Me)Orn for hCMEC/D3.

Extended Data 1: Reproducible code for regression.

Extended Data 2: Regression data.

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Project administration, JB, JW, MS; Resources, JB, JW, PU, JCS; Supervision, CB, JW, MS, JB, GB; Validation, CB, MS, JW, GB, JB, CS; Visualization, CB, CS; Writing – original draft, CB; Writing – review & editing, JB, JW, MS, GB, CS, EM, PU, JCS, MK, JC.

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Availability of data and materials: The data supporting the conclusions of this article is included within the published article. The data for the regression curve is made available with this manuscript in extended data file 2, together with an in-detail reproducible R Markdown file in extended data 1.

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List of abbreviations: ACN: acetonitrile; AGM™: Astrocyte Growth Medium with all supplements; AJ: adherens junctions; ALK: anaplastic lymphoma kinase; AUC: area under the curve; BBB: blood brain-barrier; BCEC: brain capillary endothelial cells; BCRP: breast cancer resistance protein; BSA: bovine serum albumin; BTB: blood-tumor barrier; Cas3: cleaved caspase 3; clogP: calculated logP; CLSM: confocal laser scanning microscopy; CNS: central nervous system; CID: collision-induced dissociation; D-Tyr(Me)Orn: D-O-methyl-tyrosine and D-ornithine; DMSO: dimethyl sulfoxide; EGM™: Endothelial Growth Medium 2 with all supplements; EDTA: ethylenediamine tetraacetic acid; EGTA: ethylene glycol tetraacetic acid; ERK1/2: extracellular signal regulated kinase; ESI: electrospray ionization; FA: formic acid; FBS: fetal bovine serum; FGFR: fibroblast growth factor receptor; HBSS: Hanks Balanced Salt Solution; HCl: hydrochloric acid; HHBSS: HBSS with 0.01 M HEPES; IF: immunofluorescence; IS: internal standard; $K_{CA}3.1$: calcium activated potassium channel; LDH: lactate

dehydrogenase; LPR-1: low density lipoprotein receptor-related protein 1; LLOQ: lower limit of quantification; MAPK: mitogen-activated protein kinase; MRM: selected reaction monitoring; mTOR: mechanistic target of rapamycin; MW: molecular weight; NaOH: sodium hydroxide; NTRK: neurotrophic tyrosine receptor kinase; MALDI: matrix-assisted laser desorption/ionization; MRP2: multidrug resistance associated protein 2; MW: molecular weight; NAM: New Approach Methodologies; NFLU: norfluoxetine; PARP: poly(ADP-ribose)-polymerase; P-gp: P-glycoprotein; PBS: phosphate-buffered saline; PM: Pericyte Medium; QC: quality control; QQ plot: quantile-quantile plot; ROI: region of interest; ROS1: proto-oncogene 1; RPMI: Roswell Park Memorial Institute; SSRI: selective serotonin reuptake inhibitor; TBME: tert-butyl methyl ether; TJ: tight junctions; UPLC-MS/MS: ultra-performance liquid chromatography tandem mass spectrometry; VEGF: vascular endothelial growth factor; ZO-1: Zonula occludens 1.

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1298 **Table 1:** Used drug concentrations and the reported *in vivo* steady-state or c_{max}-concentrations

drug	Used concentration [ng/mL] [µM]	Steady-state/c_{max} concentration [ng/mL]
Fluoxetine	150 | 50 | 15 0.48 | 0.16 | 0.048	Human steady state 25.1; 215 (~50 % NFLU) [98,99]
Futibatinib	300 | 100 | 30 0.72 | 0.24 | 0.072	Human c_{max} ~ 100 [100]
Larotrectinib	1800 | 600 | 180 4.20 | 1.40 | 0.420	Human c_{max} in steady state 914 [101]
Lorlatinib	1500 | 500 | 150 3.69 | 1.23 | 0.369	Human c_{max} in steady state 576.5 [102]
Octreotide	100 | 10 | 1 0.098 | 0.0098 | 0.00098	Human steady state 1.56-2.97 [103]
Olaparib	50000 | 15000 | 5000 115 | 34.5 | 11.5	Human steady state c_{max} 6370 c_{max} 4740 [104,105]
Paroxetine	300 | 100 | 30 0.91 | 0.3 | 0.091	Human steady state 50.1 [99]
Repotrectinib	3000 | 1000 | 300 8.44 | 2.81 | 0.844	Human steady state 1317 [106]
Selinexor	300 | 100 | 30 0.677 | 0.226 | 0.0677	Human c_{max} 693 [107]
Selitrectinib	15000 | 5000 | 1500 39.47 | 13.16 | 3.947	Mice c_{max} 6623 [108]
Senicapoc	2000 | 666.67 | 200 6.19 | 2.06 | 0.619	Human steady state 137 [109]
Temsirolismus	2000 | 666.67 | 200 1.94 | 0.46 | 0.194	Human c_{max} ~2500 [110]
Ulixertinib	3000 | 1000 | 300 6.9 | 2.3 | 0.69	Human c_{max} and steady state 2031 [111]

1299 NFLU = norfluoxetine; c_{max} = maximum concentration.

Table 2: Sample preparation for drug quantification

Drug	Calibration range [ng/mL]	System	Sample preparation
Fluoxetine	0.3 - 300	TQ-S1	Liquid-liquid extraction with 50 µL borat buffer (pH 9) and 700 µL TBME. After 10 min overhead shaking, 150 µL of supernatant was transferred and evaporated. Samples were redissolved in 200 µL H_2O/ACN (95/5, v/v) + 0.1% FA.
Octreotide	0.03 - 10	TQ-S2	Protein precipitation with 150 µl ACN + 0.1% FA, evaporated, redissolved in 100 µL H_2O + 0.1% FA.
Olaparib	0.1 - 300	TQ-XS	Protein precipitation with 150 µl ACN + 0.1% FA, evaporated, redissolved in 100 µL H_2O/ACN (95/5, v/v) + 0.1% FA.
Paroxetine	1 - 1000	TQ-S2	Protein precipitation with 150 µl ACN + 0.1% FA and 200 µL H_2O + 0.1% FA added.
Selinexor	0.1 - 3	TQ-S1	Protein precipitation with 100 µl ACN + 0.1% FA, evaporated, redissolved in 100 µL H_2O/ACN (95/5, v/v) + 0.1 % FA.
Senicapoc	1 - 3000	TQ-S1	Protein precipitation with 150 µl ACN + 0.1% FA, evaporated, redissolved in 100 µL H_2O/ACN (95/5, v/v) + 0.1 % FA.
Temsirolismus	0.1 - 1000	TQ-S1	Protein precipitation with 150 µl ACN + 0.1% FA and 200 µL H_2O + 0.1% FA added.
Ulixertinib	0.1 - 100	TQ-S2	Protein precipitation with 150 µl ACN + 0.1% FA and 200 µL H_2O + 0.1% FA added.
Multimethod for tyrosine kinase inhibitors (TKI)			
Futibatinib	0.03 - 30	TQ-S1	Protein precipitation with 100 µl ACN + 0.1% FA, evaporated and redissolved in 100 µL H_2O/ACN (95/5, v/v) + 0.1% FA.
Larotrectinib	0.01 - 10		
Lorlatinib	0.03 - 30		
Repotrectinib	0.01 - 10		
Selitrectinib	0.01 - 10		

Table 3: Reported *in vivo* brain-to-plasma ratio of the evaluated drugs

drug mean ± SD	Brain-to-plasma ratio				
Fluoxetine **15.4 ± 4.39**	15 ± 3 human[m], B/P_{SS} 112	10 ± 6 human[m], B/P_{SS} 113	21.2 ± 16.2 human[m], B/P_{SS}[c] 114	2.6 ± 1 human[m], B/P_{SS}[c] 115	
Futibatinib **0.17 ± 0.05**	~0.17 ± 0.05 mice [BT], B/P_{2h} 116				
Larotrectinib **0.06 ± 0.012**	0.036 ± 0.01 mice, B/P_{4h}	0.05 ± 0.02 mice, B/P_{1h} 117	0.09 ± 0.03 mice, B/P_{8h}		
Lorlatinib **0.63 ± 0.084**	0.734 ± 0.124 human[z.T. BM], $CSF/P_{uu,SS}$[c] 102,118,119		0.7 ± 0.2 mice, B/P_{AUC} 120,121	0.46 ± 0.06 mice, B/P_{8h} 122	
Octreotid **0**	0 mice 123				
Olaparib **0.03 ± 0.008**	0.026 macaques, $K_{p,uu,brain}$	0.041 macaques, $K_{p,brain}$ 95	0.036 mice [BM], $K_{p,brain}$	0.022 mice [BM], $K_{p,uu,brain}$ 96	0 rat
Paroxetine **0.97 ± 1.59**	4.27 ± 2.64 human, B/P_{ss} 124	0.35 mice, $K_{p,brain}$	0.22 mice, $K_{p,uu,brain}$ 125	0.38 mice, $K_{p,brain}$	0.25 mice, $K_{p,uu,brain}$ 0.354 mice, B/P_{AUC} 126
Repotrectinib **0.15 ± 0.06**	0.246 Human[BM], $CSF/P_{uu,SS}$[c] 106	0.15 ± 0.03 mice, B/P_{4h} 127	0.103 ± 0.011 rat $B/P_{0,5h}$ [c] 128	0.091 ± 0.023 rat B/P_{2h} [c]	
Selinexor **0.68 ± 0.084**	0.72 rat (individuals), B/P_{2h}	0.82	0.62 129	0.58 macaques (individuals), B/P_{2h}	0.65
Selitrectinib **0.04 ± 0.014**	0.021 ± 0,004 mice, B/P_{4h} 108	0.058 ± 0.042 rat $B/P_{0,5h}$ [c] 128	0.041 ± 0.01 rat B/P_{2h} [c]		
Senicapoc **4.74 ± 2.26**	~5 5×FAD mice $B/P_{n.a.}$ 130	7.32 ± 4.84 rat B/P_{1h}	7.07 ± 3.35 rat B/P_{4h} 131	2.2 ± 0.4 rat $B/P_{uu,1h}$	2.1 ± 0.4 rat $B/P_{uu,4h}$
Temsirolimus **0.72 ± 0.45**	1.43 ± 0.90 Human[BT], tumor/ blood$_{SS}$ 133	0.0023 ± 0.0082 Human[BT], CSF/P_{uu}[c] calculated with protein binding [132] 134			
Ulixertinib **0.67 ± 0.66**	0.0176 mice [PDX], B/P_{AUC} 53	1.33 ± 0.42 Human[BT], $CSF/P_{uu,ss}$ 97			

[c] = calculated from reported data; [m] = with metabolite; [BM] = with brain metastasis; [BT] = with brain tumor; [PDX] = BT40 Xenograft PDX; B/P = brain-to-plasma ratio; B/P_{uu} = unbound brain-to-plasma ratio; B/P_{AUC}= AUC-brain to AUC-plasma ~ B/P_{ss} = brain-to-plasma ratio in steady state ~ $K_{p,brain}$ = brain-to-plasma partition coefficient; $B/P_{uu,ss}$ = unbound brain-to-plasma ratio in steady state ~ $K_{p,uu,brain}$ = unbound brain-to-plasma partition coefficient; CSF/P_{uu}= CSF concentration-to-unbound plasma concentration; AUC = area under the curve.

Table 4: Selected drugs and BBB relevant characteristics

drug	Mode of action	Efflux substrate	clogP MW	Relevance to brain tumor therapy
Fluoxetine	SSRI	P-gp[135]	4.57 309.3	Xenograft[88]
Octreotide	Somatostatin analog	P-gp and mrp2[136]	2.51 1019	Negative control
Olaparib	PARP inhibitor	P-gp[96]	1.24 434.5	NCT01390571[96]
Paroxetine	SSRI	P-gp[135]	4.23 329.4	Positive control
Selinexor	Exportin 1 inhibitor	-	2.68 443.3	NCT04421378
Senicapoc	K_{CA}3.1 blocker	-	3.53 323.3	SENIPERA, EU-CT: 2025–522605-37–00[86]
Temsirolismus	mTOR inhibitor	P-gp[110]	7.46 1030	[137] NCT05773326
Ulixertinib	ERK1/2; MAPK mutations inhibitor	likely P-gp and BCRP[138,139]	4.34 433.3	NCT01781429[140] NCT03698994[141] NCT05804227[97]
TKI				
Futibatinib	FGFR inhibitor	P-gp[142]	1.96 418.5	[143]
Larotrectinib	NTRK inhibitor	P-gp and BCRP[144]	2.00 428.4	NCT02637687[145] NCT02576431[146]
Lorlatinib	ALK inhibitor	P-gp[122]	1.87 406.4	[147] NCT06333899
Repotrectinib	ALK-, ROS1-, and NTRK inhibitor	P-gp and BCRP[148]	2.96 355.4	NCT04094610[149] NCT03093116[150]
Selitrectinib	NTRK inhibitor	P-gp and BCRP[127]	1.86 380.4	NCT03215511

ALK: anaplastic lymphoma kinase; clogP: calculated logP with ChemDraw; ERK1/2: extracellular signal regulated kinase; FGFR: fibroblast growth factor receptor; K_{CA}3.1: calcium activated potassium channel; MAPK: mitogen-activated protein kinase; MW: molecular weight; mTOR: mechanistic target of rapamycin; NTRK: neurotrophic tyrosine receptor kinase; PARP: poly(ADP-ribose)-polymerase; ROS1: proto-oncogene 1; SSRI: selective serotonin reuptake inhibitor.

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