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1 **Multiscale modelling of drug transport and metabolism in liver spheroids**

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1 **Abstract**

2 In early preclinical drug development, potential candidates are tested in the laboratory using
3 isolated cells. These *in-vitro* experiments traditionally involve cells cultured in a two-
4 dimensional monolayer environment. However, cells cultured in three-dimensional spheroid
5 systems have been shown to more closely resemble the functionality and morphology of cells
6 *in-vivo*. While the increasing usage of hepatic spheroid cultures allows for more relevant
7 experimentation in a more realistic biological environment, the underlying physical processes
8 of drug transport, uptake and metabolism contributing to the spatial distribution of drugs in
9 these spheroids remain poorly understood. The development of a multiscale mathematical
10 modelling framework describing the spatiotemporal dynamics of drugs in multicellular
11 environments enables mechanistic insight into the behaviour of these systems. Here, our
12 analysis of cell membrane permeation and porosity throughout the spheroid reveals the
13 impact of these properties on drug penetration, with maximal disparity between zonal
14 metabolism rates occurring for drugs of intermediate lipophilicity. Our research shows how
15 mathematical models can be used to simulate the activity and transport of drugs in hepatic
16 spheroids, and in principle any organoid, with the ultimate aim of better informing
17 experimentalists on how to regulate dosing and culture conditions to more effectively
18 optimise drug delivery.

19

20 **Keywords:** Drug transport; Spheroid; Organoid; Hepatocytes; Systems pharmacology;
21 Mathematical modelling.

1 **1 Introduction**

2 The discovery of potential toxicity *in-vitro* remains an important process in providing pre-
3 clinical safety assurances during drug development. However, conventional 2D *in-vitro*
4 experiments, such as monolayer cell culture, tend to be poorly predictive of toxicity, and
5 emerging 3D systems are shown to be more physiologically relevant and predictive of the *in-*
6 *vivo* environment (1, 2). Accordingly, 3D cell culture systems such as multicellular spheroids
7 are increasingly being utilised in drug development and hepatic safety assessment (3, 4).
8 Although 3D spheroid systems offer improvements in terms of physiological relevance and
9 *in-vivo*-like functionality, the mechanistic interaction between these systems and drugs is not
10 yet fully understood.

11 Multiscale *in-silico* methods can improve the application of 3D spheroid models to assess the
12 hepatotoxicity of drug candidates (5, 6). Indeed, mechanistic mathematical modelling of drug
13 metabolism and transport in 3D microtissues is important for the pharmaceutical industry as
14 it facilitates an improved platform for both preclinical drug development and *in-vivo*
15 extrapolation (7). This utilisation of mathematical models, devised to tackle pharmacological
16 research challenges in a systems biology approach, has become known as part of the evolving
17 field(s) of systems pharmacology and/or systems toxicology (8, 9). This approach is a
18 multiscale, multidisciplinary field that employs holistic, integrative methods in order to
19 enhance the understanding and prediction of emergent system properties. Moreover, this
20 methodology is strictly quantitative requiring the integration of quantitative data and
21 modelling to develop mechanistic knowledge of the system and reveal pharmacological and
22 toxicological properties. Consequently, systems pharmacology models are becoming an

1 increasingly important part of the toolkit to improve capabilities and drive innovation for *in-*
2 *vitro* safety assessment (10-12).

3 In this study we have characterised the spatiotemporal dynamics of drugs in an *in-vitro*
4 hepatic spheroid system by simulating relevant physical processes *in-silico*. A data-driven,
5 multiscale, mathematical modelling framework combining mechanistic information relating
6 to the diffusion, transport and metabolism of chemical species in a hepatocyte spheroid is
7 presented. A microscale single-cell model is analysed to study different transport mechanisms
8 by varying boundary conditions on the cell membrane. This model is then coupled to a
9 multicellular model developed to evaluate the effects of cellular arrangement and density on
10 the transport and penetration of drugs, simulating the *in-vitro* microtissue environment. Such
11 effects include a non-linear relationship between drug lipophilicity and spheroid penetration
12 whereby drug delivery to the spheroid core is minimised for drugs of intermediate
13 lipophilicity. The integration of experimental data allows for the development of realistic
14 geometries and parameterisation of the multiscale model for a range of drugs. Ultimately, by
15 accurately simulating the processes of drug transport and metabolism we aim to enhance the
16 understanding of underlying mechanisms and optimise the use of these systems *in-vitro*.

17

18 **2 Methods**

19 ***2.1 Microscale transport – crossing the cell membrane***

20 To simulate the distribution of drugs throughout a 3D tissue comprised of multiple
21 hepatocytes, it is necessary to determine how drugs penetrate and cross the cell membrane.
22 This membrane comprises a phospholipid bilayer, providing a hydrophobic protective barrier

1 for the cell. Consequently, this chemical barrier property is a key determinant in the effective
2 permeability of any drug. Many factors affect drug permeability in tissues such as ionisation,
3 aqueous diffusion between lipid barriers, and protein binding, but the partition into the
4 membrane (determined by lipid solubility) is one of the most important (13). Highly
5 lipophilic substances can more readily penetrate the membrane via free diffusion, whilst
6 relatively hydrophilic substances, (highly soluble in polar solvents such as water or blood),
7 cannot enter the cell easily and require specific transporters (Figure 1A). The relative role of
8 transporter proteins in intracellular drug transport is still debated and there remain different
9 views as to whether passive diffusion or carrier-mediated transport is the major mechanism
10 (14-18). For the entirety of this study we refer to the two main types of transport: passive
11 diffusion – entering cells down a concentration gradient directly through the membrane
12 (passive); and carrier-mediated transport – entering cells via specific transporter proteins
13 embedded in the plasma membrane (passive or active).

14 The mathematical representation of microscale drug transport across a cell membrane can be
15 studied with a simple model considering the processes governing drug concentration
16 dynamics in two phases, inside and outside the cell, with a permeable barrier in-between.
17 Once inside the cell, the drug is removed via metabolism. We assume diffusion occurs at
18 different rates inside (D_I) and outside (D_E) of the cell, which we initially assume is spherical
19 of radius R , but relax this assumption in section 2.3. The drug concentration (C) dynamics
20 inside the cell are given by the partial differential equation (PDE):

$$\frac{\partial C}{\partial t} = D_I \nabla^2 C - \frac{V_{max} C}{C + K_m}, \quad (1)$$

1 where V_{max} is the maximum metabolic rate and K_m represents the drug concentration at
 2 which metabolism is half maximal. Since there is no flow within the *in vitro* system, and the
 3 dominant form of removal within the multiscale model is assumed to be due to intracellular
 4 metabolism, we assume that outside the cell drug transport is governed by diffusion processes
 5 only:

$$\frac{\partial C}{\partial t} = D_E \nabla^2 C . \quad (2)$$

6 For simplicity, we assume that the problem is radially symmetric and rescale the model with
 7 respect to cell radius and internal diffusion time (such that the cell boundary is now given by
 8 $r = 1$) to give

$$\frac{\partial C}{\partial t} = \frac{1}{r^2} \frac{\partial}{\partial r} \left(r^2 \frac{\partial C}{\partial r} \right) - \frac{V_{max} C}{C + K_m}, \quad r \leq 1, \quad (3)$$

$$\frac{\partial C}{\partial t} = \frac{D}{r^2} \frac{\partial}{\partial r} \left(r^2 \frac{\partial C}{\partial r} \right), \quad r > 1, \quad (4)$$

9 where $D = D_E/D_I$ due to rescaling (see supplementary material for details). We impose the
 10 following boundary conditions at the cell centre ($r = 0$), for radial symmetry, and a distance
 11 away from the cell ($r = r_{max}$):

$$\frac{\partial C}{\partial r} = 0, \quad r = 0, \quad (5)$$

$$C = C_{r_{max}}, \quad r = r_{max}, \quad (6)$$

12 where $C_{r_{max}}$ is a constant supply term. Assume that the flux at the cell boundary is equal such
 13 that mass is conserved, i.e.,

$$D_I \frac{\partial C_I}{\partial r} = D_E \frac{\partial C_E}{\partial r}, \quad r = 1, \quad (7)$$

1 where C_I and C_E are used to distinguish between interior and exterior drug concentrations at
2 the cell membrane boundary. A further boundary condition must be specified at the cell
3 membrane boundary in order to solve the coupled PDE system and investigate the effects of
4 different means of drug transport.

5 2.1.1 *Passive diffusion*

6 The following boundary condition is imposed to describe the flux of drug into the cell due to
7 passive diffusion:

$$D_I \frac{\partial C_I}{\partial r} = D_E \frac{\partial C_E}{\partial r} = Q(C_E - C_I), \quad r = 1, \quad (8)$$

8 where Q is the permeability coefficient. The mathematical model can be solved numerically
9 in MATLAB R2017b. For methodological details regarding derivations, numerical solutions
10 and simulations of microscale transport, see the supplementary material.

11 The impact of the permeability coefficient, Q , on the steady-state distribution of drug
12 concentration can be seen in Figure 1B (for temporal dynamics, see supplementary
13 animations). For low permeability coefficients ($Q \ll 1$), there is less drug penetration per unit
14 time and so there is a low steady-state value inside the cell and a large discontinuity at the
15 cell membrane. As Q increases, relatively more drug enters the cell per unit time and in the
16 limit, as $Q \rightarrow \infty$, the steady-state solutions converge such that the drug concentration profile
17 is continuous ($C_E = C_I$) at the cell membrane boundary (which now provides no effective

1 barrier or resistance) and the steady-state profile represents the balance of supply via
2 diffusion and removal via intracellular metabolism.

3 2.1.2 Carrier-mediated transport

4 For drugs whose physicochemical properties prohibit direct permeation across the cell
5 membrane, specific transporter proteins are required that can mediate the transfer process.
6 The reliance on transporter (or carrier) proteins dictates that the flux is now saturable with an
7 explicit dependence on the surface area concentration, binding affinities, and activity of
8 transporters in the cell membrane. In this scenario, the boundary condition representing
9 membrane transport cannot be sufficiently represented by the passive diffusion condition in
10 equation (8) and so we implement a simple carrier model as applied in other similar
11 physiological membrane transport models, e.g., Keener & Sneyd (19) and Wood & Whitaker
12 (20). This carrier model can be applied to define the flux boundary condition for the carrier-
13 mediated transport model scenario:

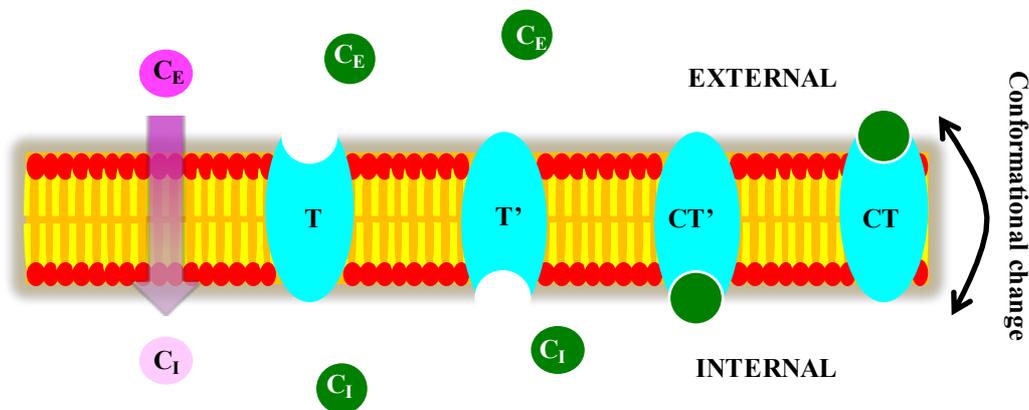
$$D_I \frac{\partial C_I}{\partial r} = \frac{T_0(C_E - \alpha_1 C_I)}{\alpha_2 + \alpha_3 C_E + \alpha_4 C_I + \alpha_5 C_E C_I}, \quad r = 1, \quad (9)$$

14 where T_0 represents transporter protein concentration on the cell membrane and $\alpha_1, \alpha_2, \alpha_3,$
15 α_4, α_5 represent algebraic expressions dependent on kinetic rates in the carrier model such as
16 binding rates (see supplementary material for more information).

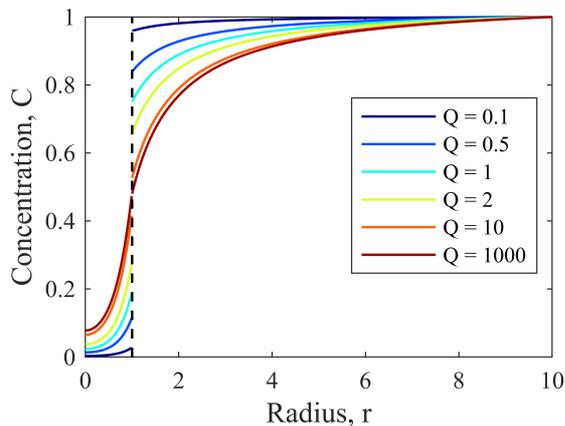
17 The barrier effect provided by the carrier-mediated transport of drugs across the cell
18 membrane allows for a discontinuity in the steady-state profile of the drug concentration
19 distribution when there is a constant external supply that diffuses towards a metabolically
20 active cell (as before with the passive diffusion case with low permeability). Indeed the

1 carrier mediated transport condition can be reduced to the passive diffusion condition
 2 mathematically with appropriate parametrisation (e.g., $T_0 = Q, \alpha_{1,2} = 1, \alpha_{3,4,5} = 0$).
 3 Furthermore, the flexibility of the carrier-mediated condition facilitates the implementation of
 4 implicit active processes whereby the flux of drug can move uphill against its concentration
 5 gradient (e.g., see Figure 1C). This can be achieved with appropriate parameterisation of the
 6 simple carrier model such that $\alpha_1 < 1$, e.g. when binding affinity/dissociation in the interior
 7 is lower/higher than exterior binding/dissociation.

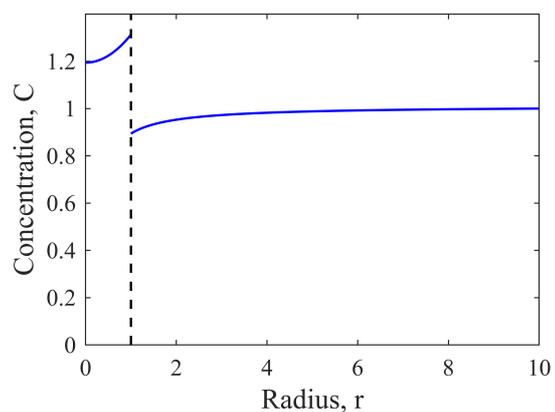
A



B



C



8 Figure 1: **Modelling transmembrane transport in a single cell.** (A): Drug transport schematics
 9 across the cell membrane. Two modes of transport are considered, passive diffusion (pink
 10 substrates/circles) and carrier-mediated transport (green substrates/circles). Drugs that permeate the
 11 cell via passive diffusion move down a concentration gradient directly through the membrane and are
 12 limited by their associated permeability coefficient. This coefficient is dependent on the

1 physicochemical properties of the drug and drugs which cross the membrane via this mechanism are
2 typically small and lipophilic. Other drugs may require the action of specific membrane-bound
3 transporter proteins to enter the cell (carrier-mediated transport). In this study, it is assumed that this
4 mechanism is dependent on carrier proteins/receptors (depicted in cyan) which can reversibly bind to
5 the substrate and undergo conformational changes to transport the substrate across the membrane.
6 Species within the figure are annotated with symbols related to mathematical models described in the
7 main text and supplementary material. (B): Variation in the permeability coefficient determines the
8 steady-state concentration profile of drug concentration in a single cell for the passive diffusion
9 transport mechanism. Low permeability results in a discontinuity at the cell membrane ($D =$
10 $2, V_{max} = 10, K_m = 0.5, C_{r_{max}} = 1$). (C): For specific parameter choices within the carrier-mediated
11 transport model, a steady-state can be reached such that the drug is transported against its
12 concentration gradient, implicitly simulating an active process ($D = 2, V_{max} = 1, K_m = 0.5, C_{r_{max}} =$
13 $1, T_0 = 1, \alpha_1 = 0.5, \alpha_2 = 1, \alpha_3 = 0, \alpha_4 = 0, \alpha_5 = 0$). Full spatiotemporal dynamics can be found for
14 (B) and (C) in supplementary animations.

15

16 **2.2 Parameterisation**

17 For the full multiscale model, describing the transport and metabolism of drugs in a
18 multicellular *in-vitro* environment, it is useful to include quantitative, dimensional parameter
19 values based on experimental data to directly represent the laboratory scenario for drugs with
20 a range of physicochemical properties. Therefore, it is important to identify relevant
21 parameter ranges for the microscale model before upscaling the problem to the
22 multicellular/tissue level by introducing hepatic spheroid geometry. There are currently three
23 key processes that determine drug dynamics in our system and require parameterisation:
24 diffusion, metabolism and [permeation](#). For simplicity and more general applicability, we will
25 focus on the passive diffusion case and not cover carrier mediated transport during analysis of
26 the multicellular model.

27 **2.2.1 Diffusion of small molecule drugs**

28 Most drugs, and nearly all drugs that cross the cell membrane via passive diffusion, are
29 categorised as small molecule drugs. These are low molecular weight compounds and

1 comprise most drugs on the market today (21). For a sample data base of 321 such drugs
2 (22), we calculated diffusion coefficients based on physical measurements of weight and
3 density (MW \sim 100-1,200Da; density \sim 0.6-2.6g/m³). Thus we propose the feasible diffusion
4 coefficient range of approximately 5×10^{-10} to 1×10^{-9} m²/s (further information in the
5 supplementary material). This narrow range supports the assertion that the main determinants
6 of drug disposition are the ability to translocate across hydrophobic diffusion barriers
7 (permeability) and chemical transformation (metabolism), while variations in the aqueous
8 diffusion rate have only minor effects on overall pharmacokinetics (13). A representative
9 value of 7.5×10^{-10} m²/s for both D_I and D_E will be considered as default for further
10 simulations.

11 2.2.2 Permeability as a function of lipophilicity

12 The permeability of a drug transported via passive diffusion is related to its lipophilicity, a
13 measurable physicochemical property that can be used to define our permeability coefficient,
14 Q . Menochet et al. (23, 24) discovered a log-linear relationship for hepatic uptake between
15 passive diffusion clearance, P_{diff} , and lipophilicity,

$$\log P_{diff} = 0.6316 \times \text{Log}D_{7.4} - 0.3143, \quad (10)$$

16 where P_{diff} has units of $\mu\text{L}/\text{min}/10^6\text{cells}$ and $\text{Log}D_{7.4}$ is a partition coefficient measure of
17 lipophilicity at a physiologically relevant pH (pH = 7.4). This relationship allowed us to
18 derive, Q , as a function of P_{diff} , and the radius of the cell, R , by taking into account passive
19 uptake across the whole cell membrane of surface area $4\pi R^2$:

$$Q = \frac{P_{diff}}{4\pi R^2} = \frac{1}{10^6} \frac{10^{(0.6316 \times \text{LogD}_{7.4} - 0.3143)}}{4\pi R^2}. \quad (11)$$

1 For the full derivation see the supplementary material. $\text{LogD}_{7.4}$ values between 1 and 5 are
 2 considered within this study to represent relatively lipophilic, small-molecule drugs (relevant
 3 for passive diffusion), with $\text{LogD}_{7.4} = 3$ as default.

4 2.2.3 Simplified drug metabolism in hepatocytes

5 Metabolism represents the principal sink/removal term in our model and the metabolic rate is
 6 likely to vary greatly depending on the chemical makeup of the drug of study, as well as the
 7 quantity and activity of metabolising enzymes present. Therefore this term is likely to have a
 8 significant impact on the overall disposition of drug concentration in a metabolically active
 9 *in-vitro* spheroid system. [Metabolic rates are assumed to be independent of space in the](#)
 10 [model for simplicity, although zonal variation may exist.](#) Brown et al. (25) reported kinetic
 11 parameters for a range of compounds to predict metabolic clearance by using cryopreserved
 12 human hepatocytes. This publication provided pharmacologically feasible V_{max} (5×10^{-6} to
 13 $4.5 \times 10^{-1} \text{ mol/m}^3/\text{s}$) and K_m (5×10^{-4} to $1.4 \times 10^{-1} \text{ mol/m}^3$) ranges for drugs primarily metabolised
 14 in the liver and were thus used as conservative guidance for this model parameterisation,
 15 given that cells cultured in 3D often display improved drug metabolism functions. As default,
 16 we consider parameters values of $V_{max} = 5 \times 10^{-3} \text{ mol/m}^3/\text{s}$ and $K_m = 1 \times 10^{-2} \text{ mol/m}^3$.

17 2.3 Macroscale – hepatocyte spheroid geometry

18 The impact of the hepatic spheroid environment on drug transport is considered by upscaling
 19 our microscale model to consider multiple discrete cells in a realistic spheroid geometry
 20 within an extracellular space (culture medium). This hepatocyte spheroid geometry was

1 generated based upon histological staining of hepatic spheroids to provide representative cell
2 sizes, number, and arrangement thereby replicating the *in-vitro* scenario within the multiscale
3 mathematical model.

4 2.3.1 *Mathematical description of spheroid geometry*

5 Histological staining of a hepatocyte spheroid revealed the spatial distribution of the cell
6 nuclei within a section (Figure 2A). This spatial information, as well as the spheroid
7 boundary, was quantified digitally with WebPlotDigitizer (26) and imported into MATLAB.
8 Due to the abundant expression of extracellular matrix in the hepatic spheroid histological
9 images, it was not possible to visualise and/or quantify the location of the hepatocyte
10 membranes. Therefore, we estimated the location of cell boundaries using Voronoi
11 tessellation (Figure 2B). Briefly, Voronoi tessellation involves assigning regions to each
12 nucleus such that any point in space within that region is closer to that nucleus than any other.
13 The boundaries of these regions can be determined by drawing perpendicular bisectors
14 between adjacent pairs of nuclei. This technique has been shown to provide viable estimates
15 for the qualitative morphology of cells in a tissue (27).

16 Cellular ultrastructure was visualised by transmission electron microscopy (TEM). TEM
17 revealed that the space between hepatocytes was narrow ($\sim 0.1\text{-}0.5\mu\text{m}$, see Figure 2C). These
18 values are supported by the literature which states intercellular spaces from 100nm to the μm -
19 scale (28, 29). Furthermore, it should be noted that fixation methods can shrink such
20 morphological features (30) and therefore we consider both narrow and wide intercellular
21 space geometries. This was achieved by contracting the vertices of each model cell towards

1 the cell's respective centre of mass by 1% ("narrow", $\sim 0.2\mu\text{m}$) or 10% ("wide", $\sim 2\mu\text{m}$)
2 (Figure 2D).

3 2.3.2 *Experimental methods*

4 Primary rat hepatocyte spheroids with an initial seeding density of 5,000 cells were produced
5 using the liquid-overlay technique as described by Kyffin et al. (31). After 11 days in culture
6 the spheroids were washed in PBS, fixed in 4% paraformaldehyde and subjected to routine
7 histological processing before staining with haematoxylin or processed for TEM analysis. For
8 TEM imaging, spheroids were fixed in 3% glutaraldehyde and processed as previously
9 described (31). Ultrathin ($\sim 70\text{--}90\text{nm}$) sections were examined using a FEI Tecnai
10 Transmission Electron Microscope at an accelerating voltage of 80Kv and images taken using
11 a Gatan digital camera.

12 2.3.3 *Numerical simulation*

13 The finite-element simulation software, COMSOL Multiphysics[®] 5.3, was used to solve the
14 multiscale model PDEs. The 2D spheroid slice geometry was imported into COMSOL and
15 the PDEs were defined as before to calculate the dynamics of drug concentration, C , for two
16 separate phases (intracellular, C_I , and extracellular, C_E):

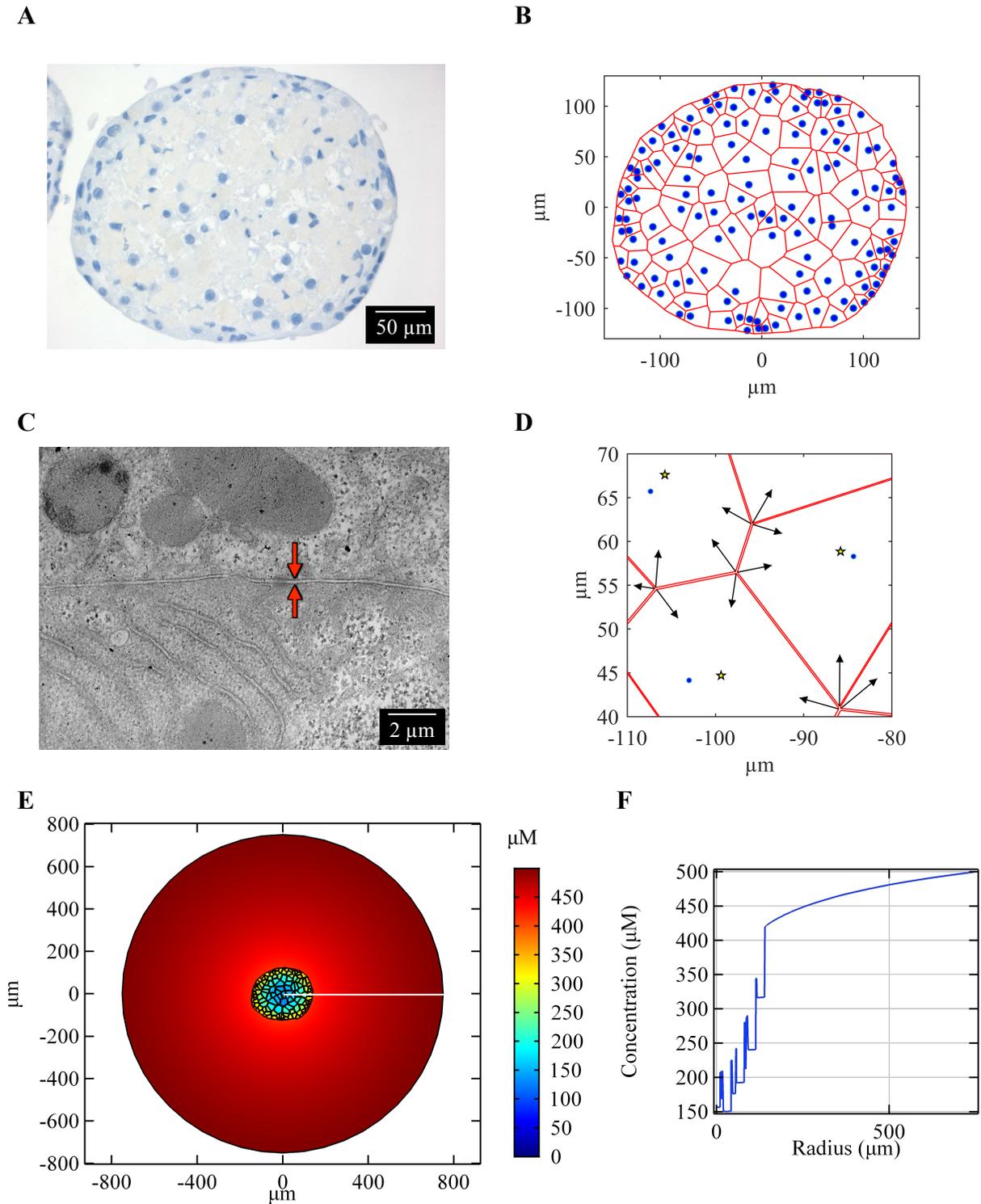
$$\frac{\partial C_I}{\partial t} = D_I \nabla^2 C_I - \frac{V_{max} C_I}{C_I + K_m}, \quad C = C_I, \quad (12)$$

$$\frac{\partial C_E}{\partial t} = D_E \nabla^2 C_E, \quad C = C_E, \quad (13)$$

17 with boundary conditions at [the every](#) cell membrane [within the spheroid](#),

$$(D_I \nabla C_I) \cdot \mathbf{n} = (D_E \nabla C_E) \cdot \mathbf{n} = Q(C_E - C_I), \quad (14)$$

1 for the general inward fluxes, where \mathbf{n} is the unit normal vector pointing out of each cell. An
2 illustrative example of the multiscale model steady-state with a constant supply of drug at the
3 outer boundary of the media phase ($C_{r_{max}} = 500\mu\text{M}$) can be seen in Figure 2E, simulated for
4 a drug with physicochemical properties based on the default parameter set described above.
5 Note that permeability Q is related to $\text{LogD}_{7.4}$ according to equation (11). A 1D cross-section
6 is plotted in Figure 2F for visualisation, highlighting the discontinuities in drug concentration
7 between intra- and inter-cellular space and the heterogeneity in drug concentration between
8 cells in different regions.



1 Figure 2: **The multiscale model including hepatocyte geometry.** (A): Histological staining of a
 2 hepatocyte spheroid slice indicating the location of cell nuclei (blue). (B): Voronoi diagram
 3 constructed to provide estimates of hepatocyte boundaries (red) based on location of hepatocyte nuclei
 4 (blue). (C): Representative TEM image of a hepatocyte spheroid showing the size of the space
 5 between adjacent cells. **The intercellular space is indicated by the red arrows.** (D): Intercellular space

1 was introduced into the model geometry by contracting the vertices of Voronoi cells (indicated by
2 black arrows) towards the centre of each cell (yellow stars; nuclei in blue). (E): Steady-state
3 distribution of an example drug ($\text{LogD}_{7.4} = 3$ with default parameters and wide intercellular space),
4 formed with a constant supply of $500\mu\text{M}$ at the outer media boundary (disc of radius $750\mu\text{m}$). The
5 drug distribution is denoted by the colour-bar, demonstrating that there are lower drug concentrations
6 in the central hepatocytes. (F): A 1D cross-section of the simulation (position indicated by white line
7 in (E)) signifies the variation of drug concentration inside and outside of the cells within the spheroid
8 structure, as well as the heterogeneity of intracellular drug concentration in different regions of the
9 spheroid.

10

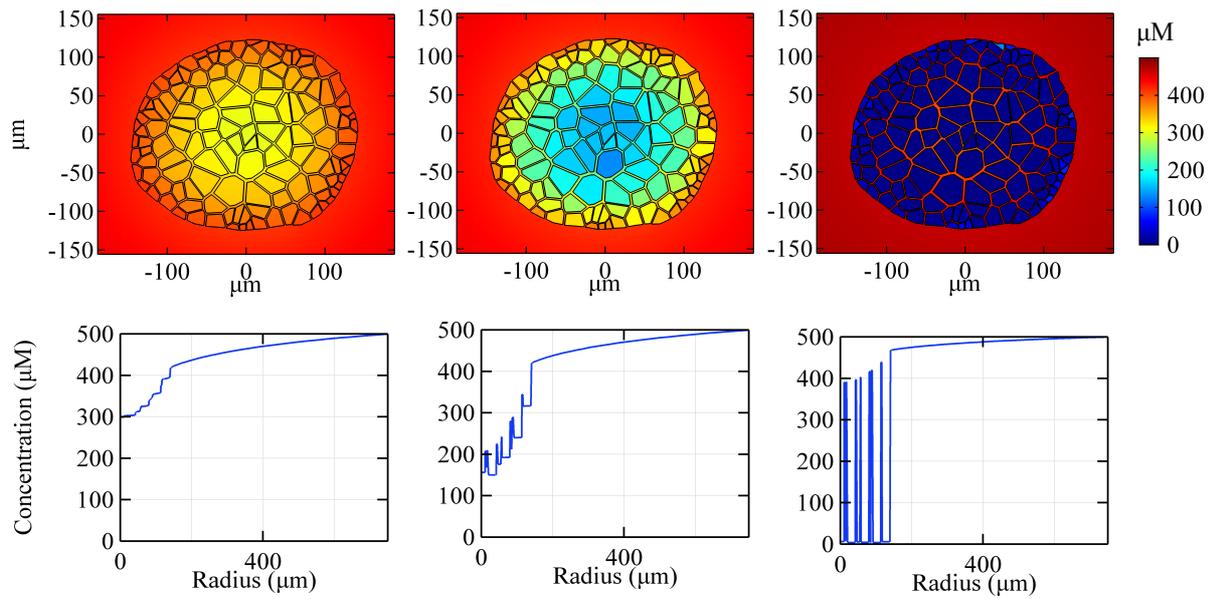
11 **3 Results**

12 **3.1 Impact of drug permeability on spatiotemporal distribution throughout spheroid**

13 The diffusion rate of a drug depends mainly on size, a property that has minimal variation in
14 small molecule drugs (a detail supported by our analysis of over 300 compounds during
15 parameterisation) and thus has relatively little impact upon drug distribution when compared
16 with the ability to translocate across the hydrophobic diffusion barrier of the cell membrane
17 (13). This translocation ability is determined by the lipophilicity of the drug during passive
18 diffusional transfer across the membrane. Therefore we consider the impact that drug
19 permeability (as determined by lipophilicity) has upon the overall dynamics within the
20 representative *in-vitro* spheroid system. This analysis is illustrated by simulating the model,
21 dosed for 3 example drugs with different permeability coefficients (corresponding to $\text{LogD}_{7.4}$
22 = 1, 3, 5, within the otherwise default parameter set) via constant supply at the external
23 boundary and comparing the steady-state spatial distribution of drug concentration (Figure 3).
24 Spatiotemporal dynamics can be found in supplementary animations.

25 The results indicate that for highly lipophilic drugs ($\text{LogD}_{7.4} = 5$), the cell membrane does
26 not represent a significant barrier to drug penetration and there is relatively little difference
27 between drug concentrations in cells and the intercellular space. For relatively lowly

1 lipophilic drugs ($\text{LogD}_{7.4} = 1$), the membranes represent a significant barrier. Drug
2 concentration is very low within the cells but relatively high in the intercellular space
3 throughout the spheroid. However, in the intermediate case ($\text{LogD}_{7.4} = 3$), there is relatively
4 little drug in the spheroid centre, both inside and outside of the hepatocytes. This is due to the
5 balance between the overall processes of drug transport towards the spheroid centre
6 (diffusion, permeability and metabolism), impacting penetration potential. Overall, it is clear
7 that an increase in permeability results in higher intracellular drug concentration but there is a
8 non-linear response in the intercellular space as permeability is increased, with a potential
9 local minimum for drugs of intermediate lipophilicity. The same observations are made for
10 narrow intercellular spaces and when varying transporter expression in the carrier-mediated
11 transport model (data not shown). This result highlights the potential importance of not only
12 permeability, but intercellular space on overall drug delivery.



PERMEABILITY

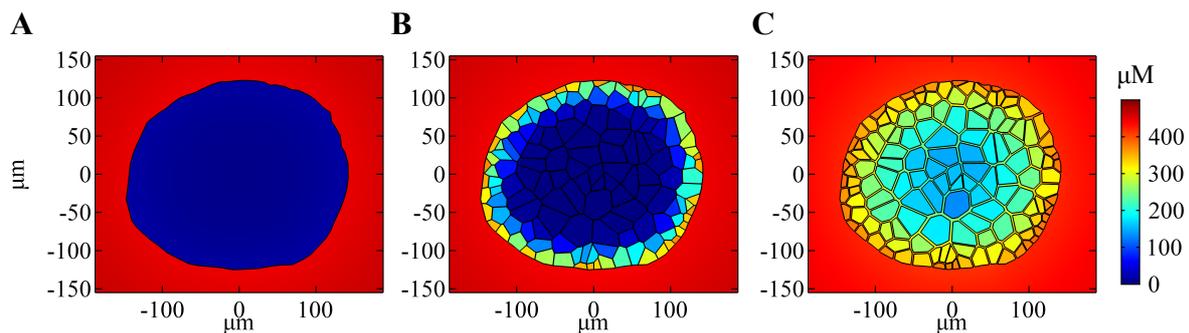
1 Figure 3: **Impact of permeability on drug dynamics distribution.** Top row: Spatial distribution of
 2 drug concentration within a hepatic spheroid for 3 different permeability coefficients (permeability
 3 decrease from left to right, $\text{LogD}_{7.4} = 5, 3, 1$). The plots represent steady-state values after constant
 4 supply of $500\mu\text{M}$ at the outer media boundary (disc of radius $750\mu\text{m}$). Bottom row: Corresponding
 5 representative 1D plots are provided for each drug using the same-cross section position as Figure 2E.

6

7 **3.2 Impact of intercellular dimensions on spatiotemporal distribution throughout** 8 **spheroid**

9 Many mathematical models of cellular spheroids consider geometrical simplifications such as
 10 radial symmetry and a homogenous continuum of cells. The consideration of a spheroid with
 11 individual hepatocytes modelled as discrete regions in space, and accompanying intercellular
 12 space, has a visible impact upon the radial drug concentration profile. This can be seen most
 13 clearly in the case of low permeability with large fluctuations in the drug concentration
 14 between intra- and inter-cellular space (Figure 3). There is a considerable range of
 15 intercellular gap sizes within spheroids, a feature which can be magnified by fixation issues

1 and cell type, with tumour spheroids notoriously exhibiting higher porosities (32). Therefore
2 it is prudent to also consider the impact of porosity (gap size) on drug delivery by simulating
3 our model for both narrow and wide intercellular space geometries, as well as a model
4 without intercellular space altogether for comparison. Steady-state spatial distributions in
5 Figure 4 suggest that intercellular space has a considerable impact upon drug penetration,
6 with increased porosity resulting in higher drug concentration for the spheroid interior.



7 **Figure 4: Impact of intercellular space on drug dynamics distribution.** Spatial distribution of drug
8 concentration within a hepatic spheroid for 3 different intercellular space geometries (no spaces (zero
9 porosity, A); narrow spaces (~0.2 μm, B); wide spaces (~2 μm, C)). The figures represent steady-state
10 values after constant supply of 500 μM at the outer media boundary (disc of radius 750 μm) with
11 default parameters.

12

13 **3.3 Translating the multiscale model to a simple continuum model**

14 From Figure 4 it is clear that, when using quantitative, measurable, microscale parameters,
15 the assumption of a homogenous continuum of hepatocytes in the spheroid will be
16 insufficient for simulating spatial drug distributions, particularly for wider intercellular
17 spaces. Therefore, we consider if there are any parameter modifications that can be made
18 such that the continuum model can be said to sufficiently replicate the simulations provided
19 by the more spatially complex discretised model. Such a model would be highly beneficial
20 for the quantification of drug dynamics with greater computational efficiency. For this

1 investigation we compare the average behaviour of the full discrete, multiscale, dimensional
 2 model (“cell-based model”) with the idealised radially symmetric, homogenised sphere
 3 model (“continuum model”) in 2D (cylindrical coordinates) given by:

$$\frac{\partial C_S}{\partial t} = \frac{D_I^{Eff}}{r} \frac{\partial}{\partial r} \left(r \frac{\partial C_S}{\partial r} \right) - \frac{V_{max} C_S}{C_S + K_m}, \quad r \leq R_S, \quad (15)$$

$$\frac{\partial C_O}{\partial t} = \frac{D_E}{r} \frac{\partial}{\partial r} \left(r \frac{\partial C_O}{\partial r} \right), \quad r > R_S, \quad (16)$$

4 where C_S and C_O represent spheroid and outer drug concentrations respectively, and $R_S = 135$
 5 μm (the average radius of the hepatocyte sphere slice in Figure 2), with boundary conditions

$$D_I^{Eff} \frac{\partial C_S}{\partial r} = 0, \quad r = 0, \quad (17)$$

$$D_I^{Eff} \frac{\partial C_S}{\partial r} = D_E \frac{\partial C_O}{\partial r} = Q^{Eff} (C_O - C_S), \quad r = R_S, \quad (18)$$

6 for effective parameters D_I^{Eff} and Q^{Eff} which represent the parameters to be modified. These
 7 parameters are logical targets for translation since they determine interior transport via
 8 internal diffusion and translocation across cell membranes in the cell-based model.
 9 Homogenisation here can be thought of as an extreme modification of the spheroid structure
 10 such that we reduce the system to a very large single cell with a single permeable membrane.
 11 The effective parameter values of the continuum model were optimised to fit the average
 12 behaviour of the cell-based models for both intercellular space geometries and a
 13 physicochemically relevant range of permeability coefficients (corresponding to $\text{LogD}_{7.4} = 1,$
 14 2, 3, 4, 5). For information regarding parameter optimisation, see the supplementary
 15 material.

1 The required modifications of effective parameters, both collectively and individually as
 2 functions of drug lipophilicity and intercellular space, are summarised in Figure 5, as well as
 3 corresponding error metrics. A combined parameter change metric in Figure 5E is introduced
 4 to quantify the relative amount of modification required for each scenario (intercellular width
 5 and lipophilicity) and defined as

$$\Delta P = \sqrt{\left(\frac{D_I^{Eff} - D_I}{D_I}\right)^2 + \left(\frac{Q^{Eff} - Q}{Q}\right)^2}. \quad (19)$$

6 From Figure 5 it is clear that ΔP is dominated by relative changes in the effective
 7 permeability coefficient, Q^{Eff} (compare Figure 5E with Figure 5A-B). Permeability must be
 8 increased to account for the intercellular space in the cell-based models (all lipophilicities),
 9 i.e., $Q^{Eff}/Q \geq 1$ for all $\text{LogD}_{7.4}$ (Figure 5B). This effectively makes the spheroid boundary
 10 in the continuum model more porous (virtually simulating gaps between cells) and the
 11 discontinuity at the spheroid boundary is reduced. It should be noted that in the dimensional
 12 cell-based models, while D_I remains constant throughout all simulations, Q will change
 13 dependent on $\text{LogD}_{7.4}$ (recall equations (10)-(11)). This is seen in Figure 5C-D with absolute
 14 changes in Q^{Eff} and Q . Permeability must be increased by a greater amount for wider
 15 intercellular spaces to be effectively simulated by the continuum model (e.g., Figure 5D) for
 16 all $\text{LogD}_{7.4}$. This is expected due to the increased porosity provided by wider gaps. Finally,
 17 effective permeability must be increased by a greater amount for low lipophilicities. This can
 18 be seen in Figure 5B where the effective permeability Q^{Eff} decreases towards the
 19 dimensional value Q with increasing lipophilicity, for both gap sizes, in a monotonic fashion.

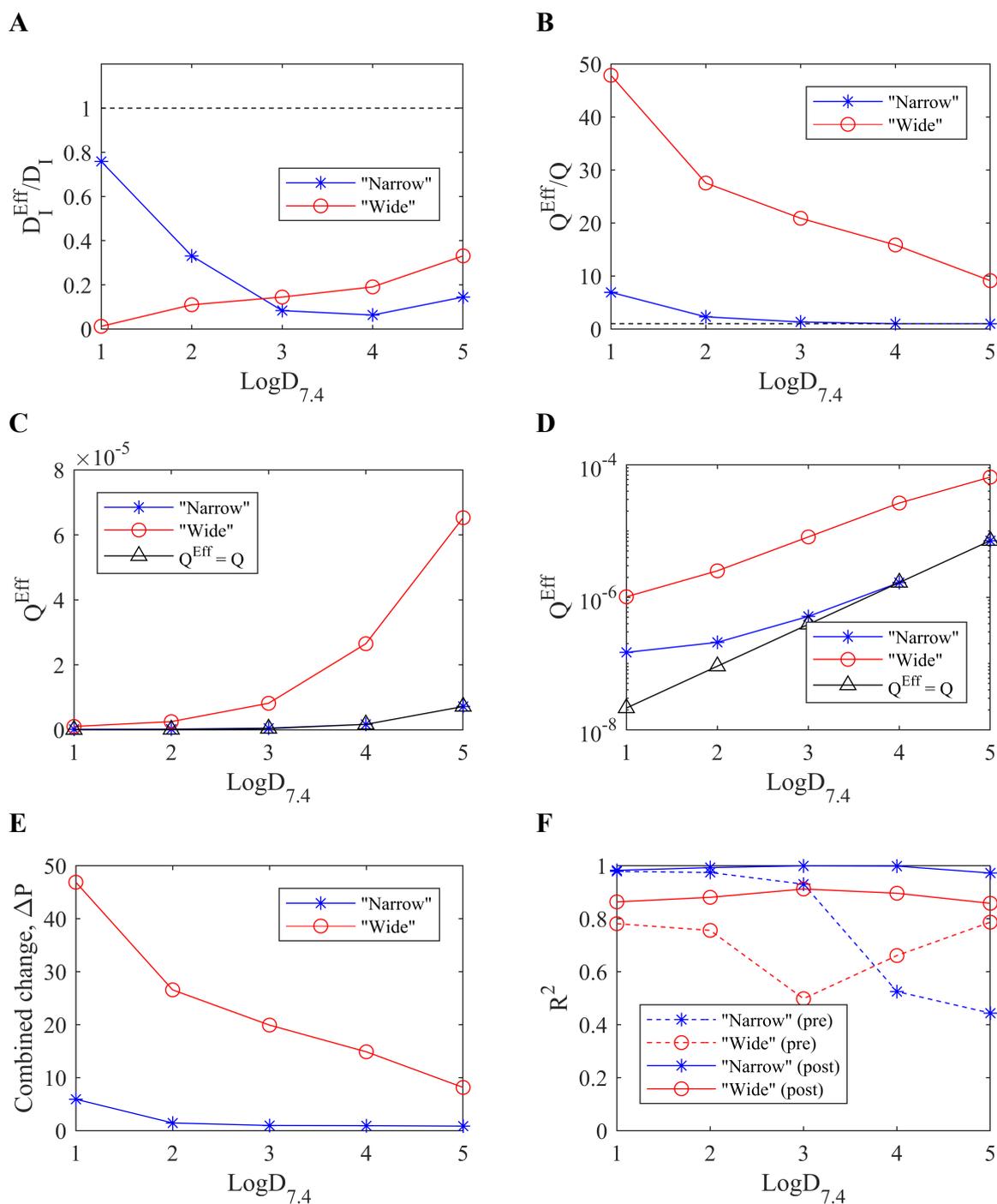
1 This reflects the increased discrepancy between transport through cells and transport between
2 cells found for drugs that are poorly lipid soluble.

3 In order for the continuum model to effectively simulate intercellular space, intracellular
4 diffusion must be decreased for all lipophilicities, i.e., $D_I^{Eff} < D_I$ for all $\text{LogD}_{7.4}$ (Figure 5A).
5 The primary effect of decreasing this parameter in the model is to increase the gradient of
6 concentrations within the spheroid. For high lipophilicity and narrow intercellular spaces, the
7 continuum model can provide a representative simulation of the cell-based model by reducing
8 D_I^{Eff} alone. This property is observed by comparing the negligible changes in Q^{Eff} relative
9 to D_I^{Eff} at high lipophilicity and narrow intercellular spaces. For example, when $\text{LogD}_{7.4} = 4$
10 and 5, D_I is decreased by 94% and 86%, while Q is unchanged (Figure 5A-B). Theoretically,
11 given a high enough value of $\text{LogD}_{7.4}$, this behaviour is expected for wide spaces too, but this
12 is beyond relevant parameter space.

13 Regardless of lipophilicity, the optimised continuum model compares better with the cell-
14 based model of narrow intercellular gaps (Figure 5F, solid lines). This is likely due to the
15 relatively lower amount of fluctuations in the mean 1D profiles as there is less extracellular
16 space in general within the spheroid. These fluctuations represent the local drug
17 concentration variation at the cellular scale due to discrepancies between intra- and extra-
18 cellular phases, which can be very high for drugs that are poorly lipid soluble (e.g., see Figure
19 3 1D profiles). Prior to any optimisation and re-scaling of dimensional parameters to their
20 effective counterparts ($D_I \rightarrow D_I^{Eff}$, $Q \rightarrow Q^{Eff}$), there was a clear pattern in the fit quality
21 between the simple continuum model approximation and the cell-based models of different
22 sized intercellular spaces (Figure 5F, dashed lines). Generally, the continuum model fits the

1 narrow spaces better for low membrane permeability and wider spaces better for high
2 permeability. This feature appears to be correlated to the overall higher intracellular drug
3 concentrations found in spheroids with wider spaces (since there is proportionally less
4 transport across metabolising cells). The (pre-optimised) continuum model exhibits very low
5 drug concentration within the spheroid at low permeability and so fits the narrow spaced
6 model better. At higher permeabilities the continuum model has relatively high interior
7 concentration and so fits the wide spaced cell-based model better (Figure S1 in the
8 supplementary material). This switch in behaviour is likely due to the continuum model only
9 providing a single barrier to permeation (spheroid boundary), which, once penetrated,
10 facilitates drug penetration via diffusion solely.

11 Interestingly, Figure 5E indicates that the cell-based model with wider intercellular spaces
12 requires more parameter modification for all drug lipophilicities. Despite the intra-spheroidal
13 gradients being vastly different between the (pre-optimised) continuum and narrow cell-based
14 model at high permeabilities (Figure S1), the boundary intracellular drug concentrations are
15 similar. Therefore the continuum model can be optimised via sufficient reduction in D_I^{Eff}
16 whilst maintaining the original permeability coefficient ($Q^{Eff} = Q$). However, in order to
17 simulate the wide cell-based model, and account for different concentrations in boundary
18 cells, a relatively greater change in Q^{Eff} was required (compare relative changes in effective
19 parameters at $\text{Log}D_{7.4} = 4$ and 5 for D_I^{Eff} and Q^{Eff} for both models, Figure 5A-B).



1 **Figure 5: Emulating the cell-based models with a simple, symmetric continuum model.** Effective
2 parameters for intracellular diffusion and permeability (D_I^{Eff} and Q^{Eff}) were optimised in the
3 continuum model to fit the output for the cell-based models for a range of drug lipophilicities. (A):
4 The optimised D_I^{Eff} value provides the required relative changes in intracellular diffusion for the
5 continuum model to match the cell-based models with narrow (blue) and wide (red) intercellular
6 space geometries. (B) Required relative changes in permeability identified by optimising Q^{Eff} .
7 Absolute values of Q^{Eff} optimised for each cell-based model and drug lipophilicity ($\text{LogD}_{7.4}$) are

1 plotted in (C). (D): Optimised Q^{Eff} on a log scale (y-axis). (E): A combined parameter change metric
2 (ΔP) indicates the summarised amount of model modification required for the continuum model to
3 effectively simulate the cell-based models. (F): Comparisons between model outputs are calculated by
4 using the R^2 error metric to determine relative quality of fits (see supplementary material for
5 definition). Comparisons are made both prior to optimisation (“pre”, direct comparison using
6 dimensional parameters, i.e., $D_I^{Eff} = D_I, Q^{Eff} = Q$) and post-optimisation (“post”).

7

8 ***3.4 Investigating the impact of permeability on the dynamic process of drug delivery in*** 9 ***different regions of the spheroid for a bolus dose***

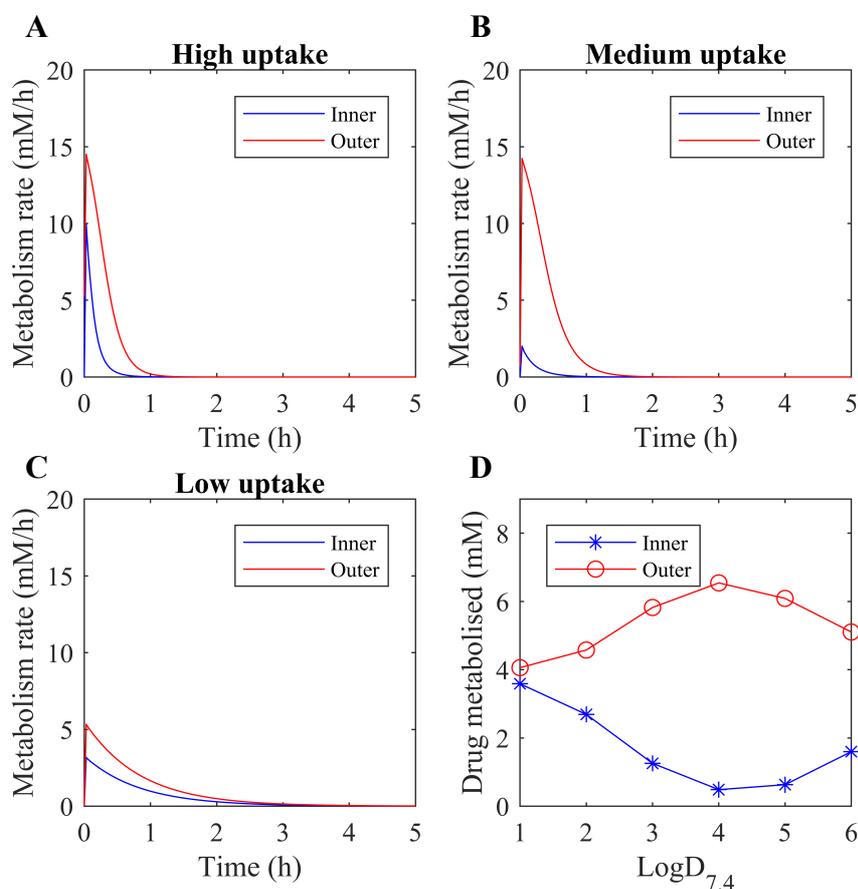
10 Intercellular space has a discernible impact on the spatiotemporal drug dynamics in the *in-*
11 *vitro* spheroid environment and moreover, a non-linear effect was revealed for local
12 concentrations within intercellular space as permeability is increased (Figure 3). Since this
13 phenomenon (i.e., a monotonic decrease in intracellular drug concentration with decreasing
14 permeability, but a non-monotonic response in the intercellular regions) cannot be described
15 by the simple continuum model, it is worth considering the potential impact of this feature on
16 drug penetration. Here we choose to examine drug delivery and subsequent effects by
17 calculating the total uptake/metabolism of the drug in different regions of the spheroid. To
18 investigate drug delivery via metabolism, we introduce the following “metabolism” variable,
19 M , with dynamics:

$$\frac{\partial M}{\partial t} = \frac{V_{max}C}{C + K_m}, \quad C = C_I, \quad (20)$$

20 which corresponds to accumulated drug metabolised and is only relevant inside model cells.
21 Corresponding model simulations are conducted with a finite bolus dose initially supplied in
22 the outer medium, uniformly distributed in the extracellular space outside the spheroid, and
23 zero-flux boundary conditions are imposed on the outer boundary of the media phase.

1 Two separate regions are defined, “outer” and “inner”, corresponding to cells of comparable
2 size in the outer boundary layer of the spheroid, $(x, y) = (-10\mu\text{m}, 110\mu\text{m})$, and the spheroid
3 centre, $(x, y) = (0\mu\text{m}, 0\mu\text{m})$. Simulations are run to the drug-free steady-state whereby all of
4 the initial dose has been removed from the system and accumulated in the effective sink
5 variable, M . For highly lipophilic drugs, the concentration dynamics are relatively similar
6 between inner and outer regions as the drug is able to be transported throughout the spheroid
7 quickly, unrestricted by permeability. However, the outer cells are exposed to slightly higher
8 concentrations and consequently more drug is metabolised in this region, demonstrated by
9 similar rates of metabolism (Figure 6A). Simulations of lowly lipophilic drugs require much
10 longer timespans in order to reach equilibrium due to the reduced uptake rate at the cell
11 membranes. However, due to the intercellular transport via diffusion, even centrally located
12 cells receive relatively high local drug exposure and metabolise at a similar rate to outer cells
13 (Figure 6C). It is the *in-silico* drugs of intermediate lipophilicity in this model scenario that
14 exhibit the most striking discrepancies between inner and outer cells (Figure 6B). The impact
15 of these varying rates of metabolism between drug lipophilicities and regions of the spheroid
16 can be evaluated by comparing the total drug metabolised (Figure 6D). The greatest
17 discrepancy in drug uptake between outer and inner hepatocytes is revealed for drugs of
18 intermediate permeability (1,250% increase from inner to outer cells for $\text{LogD}_{7.4} = 4$
19 compared to just +13% for $\text{LogD}_{7.4} = 1$ and +219% for $\text{LogD}_{7.4} = 6$). Furthermore, outer
20 cells in this case receive the most drug out of all three cases studies and the inner cells receive
21 the least (Figure 6D). This effect can potentially be exacerbated when carrier-mediated
22 transport kinetics are modelled at the cell membrane, due to the saturating effects of this
23 uptake mechanism (arbitrary transporter parameterisation, data not shown). This feature has

1 the potential to significantly impact experimental design considerations and *in-vitro* drug
2 efficacy and toxicity evaluation.



3 Figure 6: **Impact of drug lipophilicity on uptake and metabolism in different regions of the**
4 **spheroid.** Metabolism rates are plotted against time as a result of model simulations following a bolus
5 dose of 100 μ M, initially uniformly distributed in the medium for varying drug lipophilicities (LogD_{7.4}
6 = 6 (A), 4 (B), 2 (C)). (D): Total metabolised drug (after complete clearance) is compared for inner
7 and outer hepatocytes within the spheroid for a range of drug lipophilicities (LogD_{7.4} = 1-6).

8

9 4 Discussion

10 The enhanced sophistication of current cell culture methodologies due to increasing
11 advancements in scientific understanding and technological developments has allowed for *in-*
12 *vitro* studies to become more physiologically relevant. There are a range of different *in-vitro*
13 models that span varying levels of complexity, reproducibility, high-throughput potential, and

1 cost. Spheroids represent an intermediate experimental model that allows for increased
2 physiological relevance over 2D monolayers due to the 3D environment, as well as more
3 appropriate cell morphology and functionality while remaining cost-effective, consistent, and
4 easy to use (1). The subsequent prevalence of liver spheroid cultures for studying hepatocyte
5 behaviour *in-vitro* is evident and represents a key component of drug development such that
6 drug candidates can be tested for efficacy and toxic potential in a 3D environment with
7 physiological gradients (31, 33-35). ~~However, such sophisticated adaptations offered by~~
8 ~~technological advancements are often prone to rapid widespread adoption, misuse and~~
9 ~~consequent distrust due to a lack of deep mechanistic understanding of the new tool. This is~~
10 ~~related to what is sometimes known as the hype cycle for emerging technologies (36).~~ Data-
11 driven multiscale mathematical models provide an ideal platform from which to try and
12 enhance mechanistic understanding of new biotechnologies by simulating the underlying
13 physical processes. Additionally, the development of spatiotemporal data generated by 3D
14 cell imaging offers tremendous opportunities for developing, parameterising and testing
15 multiscale mathematical models and in response, mathematical modelling can be successfully
16 used to optimise these developing technologies.

17 In this study we developed a mathematical model of drug transport and metabolism in a
18 multiscale spheroid framework, accounting for microscale processes such as membrane
19 transport kinetics and how they relate to the physicochemical properties of a drug, and
20 macroscale features such as the geometry of a hepatocyte spheroid, informed by imaging
21 data. Cellular uptake of drugs was modelled by the two major processes of transport across
22 the cell membrane, passive diffusion and carrier-mediated transport (17). The carrier-
23 mediated transport microscale model was innately more complex, depending on quantities

1 such as transporter protein expression, binding kinetics and rates of conformational change
2 and this complexity allowed for a wider array of dynamic mechanisms such as enzymatic
3 saturation and active processes. The extensive parameterisation required to quantify the
4 carrier-mediated transport model depends on more compound-specific information, and so
5 the passive diffusion case became the main focus of investigations within the generalised
6 multiscale framework, more relevant for relatively lipophilic compounds.

7 The explicit representation of individual hepatocytes based on imaging data allowed for an
8 investigation into the [effects of including a distinct cell-based geometry in the model](#). The
9 [model suggests that steady-state intracellular drug concentrations increase monotonically](#)
10 [with increasing drug lipophilicity](#). However, a non-monotonic relationship was revealed
11 [between drug lipophilicity and intercellular drug concentration](#) (Figure 3), while the width of
12 the intercellular space further impacted spatial drug distribution (Figure 4). Intercellular space
13 geometry, or spheroid porosity, is therefore a key physiological feature of the multicellular
14 structure but is both difficult to accurately quantify and known to vary widely between cell
15 types. This is particularly relevant in the case of tumour-derived spheroids, whose
16 morphology tends to be more porous (32), and organoids that are increasingly being used in
17 efficacy testing for [tumour](#) cells (37). We therefore studied two different average intercellular
18 widths informed by TEM data and the literature which suggested a range of 10^2 - 10^3 nm-scale,
19 with results varying due to cell type, tumour phenotype and experimental artefacts such as
20 fixation (29, 30, 38).

21 While it is important to account for intercellular space within spheroids to correctly model
22 drug delivery, the consequent increase in complexity by modelling this feature explicitly
23 renders detailed analytic work intractable and deriving numerical solutions is costly with

1 respect to time and computational power requirements. Therefore it is appropriate to consider
2 the application of simplified models that consider averaged or homogenised system
3 behaviour and under what conditions they can provide valid approximations (39). We have
4 shown how to approximate the cell-based models using a simple, symmetric, continuum
5 model by reparameterising dimensional parameters to re-scaled effective counterparts. For
6 relatively narrow intercellular gaps, these approximations are more accurate and the required
7 parameter changes are reduced. The differences between the models, due to the explicit
8 representation of intercellular space (porosity) within the cell-based model, are largely
9 accounted for by increasing the effective permeability parameter. This parameter determines
10 the translocation of drug at the boundary between intra- and extracellular phases and, as a
11 result, the degree of discontinuity in drug concentration across the cell membrane. This is
12 particularly important at lower lipophilicities when permeability limitations are maximised.
13 For higher lipophilicities and narrow intercellular space, the spatially averaged dynamics of
14 the cell-based model can be effectively simulated with the symmetric continuum model by
15 appropriate reductions in the effective internal diffusion parameter only. Further work is
16 required to determine the impact of spatially varying quantities that might exist within a
17 spheroid such as intercellular space or transporters that vary zonally (40), and how these
18 might compare between continuum models and cell-based models. Metabolic rates are also
19 known to vary in space throughout multicellular structures due to gradients in environmental
20 factors such as oxygen and glucose (41). Alternative model simplifications that might
21 expedite analysis can be made by careful consideration of potentially redundant model
22 complexities such as intracellular diffusion, which may be neglected in some scenarios. The
23 model currently neglects any intracellular binding of the drug for simplicity, focusing on the

1 dominant mechanisms of transport and removal (metabolism) that drive the spatiotemporal
2 dynamics. However, for specific future applications of the model, intracellular binding could
3 be considered by ascertaining the relevant fraction unbound for a particular drug, as this will
4 lower the rate of metabolism for those drugs which bind strongly to intracellular proteins and
5 nuclear structures.

6 The discovery of an apparent local minimum in drug penetration, whereby intercellular
7 concentrations are lower for intermediate membrane permeation, motivated an investigation
8 into corresponding effects on drug delivery, uptake and metabolism in spheroid centres for a
9 bolus dose (Figure 6). The results of this investigation indicated that indeed, it is feasible to
10 observe minimal drug uptake at the spheroid centre for drugs of intermediate lipophilic
11 properties (with the majority of drug being metabolised at the outer regions). These
12 mechanistic insights and modelling results have potential impact for the dosing of spheroid
13 systems *in-vitro* as well as relevance for analogous *in-vivo* systems such as avascular
14 tumours. It is not necessarily sufficient to assume that increasing a chemical's lipid solubility
15 will enhance its metabolism at the spheroid centre. Lowly lipid soluble drugs may require a
16 much longer time in culture but ultimately metabolise the drug more uniformly throughout
17 the spheroid. Accounting for reduced penetration due to the intermediate lipophilic property
18 may be alleviated somewhat by increasing the dose, but this could have potentially toxic
19 consequences from overdosing cells at the spheroid boundary. Other experimental design
20 options include manipulating permeability (by chemical modification or intervention, but this
21 could potentially further increase the divergent amounts of drug being metabolised in
22 different regions of the spheroid) or using smaller spheroids. These investigations could be
23 conducted within the *in-silico* framework, in the first instance, to guide strategy. The

1 implications of drug delivery characteristics based on permeability parameters could
2 potentially be translated to targeting delivery in tissues of multiple cell types expressed
3 zonally. For example, targeting the central zone of a spheroid that contains cells of a different
4 phenotype (e.g. cancerous/hypoxic) may be aided by manipulating these properties regarding
5 permeability, i.e., making certain that the permeability is either relatively high or relatively
6 low to ensure delivery to the spheroid centre. The combination of mathematical modelling
7 with experimental imaging provides a convenient *in-silico* testing toolkit to optimise the use
8 of 3D cell culture systems in the laboratory and maximise the potential of spheroid models
9 aiding drug discovery, toxicity testing and dose optimisation.

10

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15 **Author Contributions**

16 JAL wrote the manuscript; JAK, ALH, HEC, CM, & PS performed the experiments; JAL,
17 SDW & RNB contributed to the mathematical modelling; JAL, DPW, SDW & RNB
18 designed the research. All authors read and approved the final manuscript.

19 **Declaration of Interests**

20 The authors declare no competing interests.

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