Three-dimensional models of chemotherapeutic agent transport in tumours

Summary of Ph.D. thesis

Szabolcs Modok, MD

Haematology Division, 2nd Department of Medicine and Cardiology Centre, Albert Szent-Györgyi Clinical Centre, University of Szeged

Oxford Drug Resistance Group, Nuffield Department of Clinical Laboratory Sciences, University of Oxford

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List of publications presented in the thesis

I. **Modok S**, Hyde P, Mellor HR, Roose T, Callaghan R. Diffusivity and distribution of vinblastine in three-dimensional tumour tissue: experimental and mathematical modelling. Eur J Cancer. 2006 Sep;42(14):2404-13.

II. **Modok S**, Scott R, Alderden RA, Hall MD, Mellor HR, Bohic S, Roose T, Hambley TW, Callaghan R. Transport kinetics of four- and six-coordinate platinum compounds in the multicell layer tumour model. Br J Cancer. 2007 Jul 16;97(2):194-200.

III. **Modok S**, Mellor HR, Callaghan R. Modulation of multidrug resistance efflux pump activity to overcome chemoresistance in cancer. Curr Opin Pharmacol. 2006 Aug;6(4):350-4. Epub 2006 May 11.

IV. Mellor HR, Snelling S, Hall MD, **Modok S**, Jaffar M, Hambley TW, Callaghan R. The influence of tumour microenvironmental factors on the efficacy of cisplatin and novel platinum(IV) complexes. Biochem Pharmacol. 2005 Oct 15;70(8):1137-46.

V. Alderden RA, Mellor HR, **Modok S**, Hambley TW, Callaghan R. Cytotoxic efficacy of an anthraquinone linked platinum anticancer drug. Biochem Pharmacol. 2006 Apr 14;71(8):1136-45.

VI. Alderden RA, Mellor HR, **Modok S**, Hall MD, Sutton SR, Newville MG, Callaghan R, Hambley TW. Elemental tomography of cancer-cell spheroids reveals incomplete uptake of both platinum(II) and platinum(IV) complexes. J Am Chem Soc. 2007 Nov 7;129(44):13400-1.

Presentation directly related to the thesis

VII. **Modok S**. The slow transport of vinblastine in tumour tissue models: is it a handicap or advantage? NCRI Cancer Conference, 2-5 Oct 2005, Birmingham, UK

1. Introduction

Cancer cells evade chemotherapy in many different ways and research into drug resistance mechanisms is an important driving force for anticancer drug development. Since the dawn of chemotherapy in the 1940's, many cellular resistance mechanisms have been observed, yet there are only a few agents capable of "disarming" these mechanisms. The lack of efficacy of pharmacological multidrug resistance inhibitors in clinical studies has been attributed to numerous factors and has generated a certain degree of scepticism on the role of multidrug transporters. In an effort to resolve the disparity between *in vitro* evidence and clinical observations, our research team have employed a two-pronged approach, ***i.e.*** to further clarify the structure-function relationship of multidrug transporters and to examine the effect of the three dimensional multicellular architecture of tumours on multidrug transporter function, inhibition and citotoxic drug distribution. Exploration of this latter field provides the leading theme of this thesis. Solid tumours display not only inducible unicellular mechanisms to escape cytotoxic effects but a number of inherent properties that confer resistance. The inherent, or multicellular resistance (MCR), is produced by high cell density and extensive cell-cell contact in the 3-D arrangement of cancer cells. These factors ensure slow penetration of chemotherapeutics through the solid tumour. The inadequate vascularisation results in increased interstitial pressure due to decreased drainage of extracellular fluids. The decreased filtration renders diffusion down the concentration gradient as the major driving force in achieving significant drug penetration through the avascular tumour regions. The multi cell layer (MCL) model comprises several layers of cancer cells grown on a semiporous membrane within a cylindrical culture well insert. The route for drug transport in MCL is mainly extracellular, and this can be hindered by several factors including cellular uptake and metabolism of drugs. Vital information about the relative contributions of diffusivity, convection, cellular uptake and metabolism to intra-tumoural drug kinetics is still missing. Mathematical modelling can facilitate the dissection of penetration/flux kinetics and allow the description of processes with widely applicable parameters such as diffusion coefficient.

1.1. Vinblastine transport studies

After intravenous (IV) administration vinblastine has a large volume of distribution suggesting quick absorption of the drug into the tissues. In contrast, vinblastine was the slowest of a panel of cytostatic drugs to pass through MCLs. Inhibition of P-glycoprotein (P-

gp) was shown to increase accumulation of P-gp substrates in deeper layers of spheroids and to decrease penetration of doxorubicin through MCL. However, the cell lines used in these investigations had previously been selected for high level P-gp expression. Clinical samples from drug resistant tumours are believed to display considerably lower ATP binding cassette (ABC) transporter expression. Consequently, a long-standing debate on the precise contribution of P-gp in affecting drug distribution in solid tumours *in vivo* remains unresolved. The aim of the present investigation was to provide detailed flux constants for vinblastine through DLD1 MCL with P-gp expression levels relevant to human tumour samples. The flux of sucrose was determined to provide a measure of exclusively interstitial diffusion. Furthermore, the measured diffusivity of vinblastine was used to estimate its tissue distribution.

1.2. Platinum compound transport studies

The Pt-complexes are widely used in cancer chemotherapy but unfortunately their efficacy is limited against the majority of malignancies. The reasons for the failure of this important class of chemotherapeutic agent are a combination of cellular drug resistance, toxicity and poor whole body or cellular pharmacokinetic profiles. Only a minor fraction of the plasma platinum dose will be available to form adducts with the DNA. The presence of axial ligands in the six coordinate Pt(IV) complexes results in lower activation rates, which might facilitate delivery of Pt(IV) drug to cancer cells at effective concentrations. The efficacy of Pt(II)-complexes is severely limited due to poor cellular uptake. The introduction of hydrophobic axial ligands in Pt(IV)-complexes was thought to provide a strategy to facilitate passive diffusion into cancer cells. Unfortunately, the correlation between lipophilicity and cellular uptake was weak for Pt(IV) compounds. In contrast, there was a better correlation between the redox potential and uptake of these compounds. This finding has generated a model, which proposes that the Pt(IV) drug exists in a dynamic equilibrium across the cell membrane and that the preferable rate of intracellular “aquation” and subsequent reactivity effectively traps the drug inside the cell. Yet despite the many advantageous features of Pt(IV)-complexes that have been demonstrated in monolayer cultures of cancer cells, there are few of this class of drug in clinical use. A possible explanation for this lack of translation may be the complexity of solid tumours *in vivo*, relative to the *in vitro* experimental system. Tumours *in vivo* represent complex 3-D cellular organisations with a characteristically harsh microenvironment comprising high cell density, low pH, toxic metabolites, low oxygenation and elevated interstitial pressure. Few studies have systematically examined the impact of

these and other factors on the efficacy of Pt(IV)-complexes in solid tumours or provided information on the distribution, diffusivity and flux of Pt(IV)-complexes through a solid tumour. It is known that the driving force for passive diffusion through the plasma membrane is the concentration gradient of the platinum compound. Hence increased penetration of Pt(IV)-complexes into tumour tissues, a higher local drug concentration and rapid intracellular aquation may significantly impact on the delivery of platinum compounds to nuclear DNA. The present investigation has examined the pharmacokinetic properties of a reductively activated Pt(IV) compound in the multicellular layer three-dimensional tumour model. This model enabled a quantitative analysis of the distribution and flux parameters for Pt-drugs. Moreover, a novel elemental imaging analysis by synchrotron radiation induced X-ray emission was applied to examine Pt-distribution and the degree of intacellular accumulation in multicell layers.

2. Aims

I. To set up a 3-D tumour model suitable for measuring drug diffusivity in tissue.

II. To characterise this experimental system, *i.e.* the MCLs in terms of morphology, cell proliferation and tissue hypoxia, ABC transporter expression at protein and mRNA level.

III. To utilise the MCLs and optimise conditions to make it a versatile assay of drug transport and distribution.

IV. Simultaneously mathematical modelling was pursued to facilitate quantification of results from drug transport experiments. Initially we aimed to describe the inherent characteristics of the transport system with and without the MCL.

V. Mathematical modelling was necessary to quantify drug diffusivity, distribution and cellular uptake in the MCL.

VI. Utilising the experimental and mathemathical model we aimed to describe the transport of a typical ABC transporter substrate *e.g.* vinblastine.

VII. We aimed to describe the transport of a novel six-coodinate platinum compound and compare it to that of a four-coordinate compound, which is similar to cisplatin.

VIII. The possibility to increase platinum drug penetration by increasing hydrostatic pressure was investigated.

IX. We aimed to experimentally determine drug distribution profile within the MCL and compare it to predictions of the mathematical model.

X. Finally, we aimed to mathematically model cytotoxic drug penetration and distribution in a simplified *in silico* 3-D tumour model.

3. Materials and Methods

3.1. Materials

Radiolabelled [^{14}C]-[$PtCl_2$(ethane-1,2-diamin)] (referred to as [^{14}C]-Pt(II)) and [^{14}C]-*cis,trans*-[$PtCl_2(OH)_2$(ethane-1,2-diamin)] ([^{14}C]-Pt(IV)) were synthesised according to published methods. All other materials were commercially available.

3.2. Cell lines and MCL culture

Caco2, MCF7^{WT}, NCI/ADR^{Res} and DLD1 cells were cultured as monolayers. MCLs were cultured using DLD1 cells at 3 x 10^5 cells/well density in Transwell-Col inserts in spinner flasks with RPMI 1640 medium.

3.3. Morphological characterisation of MCL

MCL went through routine histological processing and 5 μm sections were stained with H&E.

3.4. Detection of proliferating cells by Ki-67 immunohistochemistry

Paraffin sections of MCL were dewaxed and after antigen retrieval (30 s at 120°C in 10 mM Tris, 1 mM Na_2EDTA, pH 9.0 buffer) stained for Ki-67 with the MIB1 mouse antibody.

3.5. Detection of hypoxia in MCL

MCL were incubated with 200 μM pimonidazole (Hypoxyprobe-1) in culture medium for 2 hours. After routine histological processing and antigen retrieval (Pronase and Brij 35) adducts were detected by Hypoxyprobe-1 Mab1 monoclonal antibody.

3.6. Detection of mdr1 mRNA expression by real-time quantitative TaqMan RT-PCR

Total mRNA was extracted from DLD1, Caco2, MCF7^{WT} and NCI/ADR^{Res} cells from monolayer cultures and levels of *mdr1* mRNA were quantified by real-time quantitative TaqMan RT-PCR 18S rRNA was quantified as the endogenous control. Relative expression levels were calculated using the $\Delta\Delta C_T$ method.

3.7. Detection of P-gp protein expression in DLD1 monolayer cultures and MCL by western blotting

Both monolayer and MCL cultures of DLD1 cells were lysed and P-gp expression was detected by western blotting using C219 monoclonal antibody.

3.8. Determination of flux of radiolabelled compounds through MCL

Inserts with MCL were transferred into 24-well plates with 1 mL fresh medium in the wells. When the insert was moved to a new well, the medium in the donor compartment (DC) was replaced by 150 μL of fresh RPMI 1640 medium containing [^{14}C]-sucrose and [^{3}H]-vinblastine-sulphate or a [^{14}C]-labelled platinum compound. The present experimental set-up was able to eliminate convective terms since the insert was kept stable in the culture well and fluid levels in the two compartments were equal thus resulting in zero fluid pressure gradients (and therefore no convection) across the MCL. In certain experiments, to provide convective force against the concentration gradient driven diffusion, the volume of the receiver chamber (RC) was increased to 1500 μL. The reverse gradient was created by increasing the DC volume to 280 μL and keeping the RC at 1000 μL. The entire experimental apparatus was placed into an incubator to maintain standard MCL culture conditions. At specific times during the assay, the insert was moved to a new well with fresh culture medium. To measure diffusive flux of the compounds from the DC through the MCL, 800 μL samples were taken from each of the used wells. The amount of radiolabelled compound was determined by liquid scintillation counting.

3.9. Non-specific binding of radiolabelled compounds to the plastic of the transport apparatus

The non-specific binding (NSB) of compounds to the lower chamber was measured by incubating the plastic inserts in wells of a 24-well culture plate in medium containing a 9:1 ratio of cold and radiolabelled compounds. Two hours later, samples were taken from the RC to measure the amount of drug that remained in the culture medium. The amount of drug was determined by liquid scintillation counting. A similar correction was made for platinum drugs in the DC. Following completion of transport assays samples were taken from the DC and the polytetrafluoroethylene (PTFE) membranes with the MCL were excised to measure bound radioactivity. In control experiments without MCLs the PFTE membrane was excised. The percent of added [^{14}C]-Pt-complex bound to the DC was determined by subtracting the percent bound to PTFE membrane in the presence or absence of the MCL, the percent

transported to the RC and from the percent decrease in the DC at the end of the transport assay.

3.10. Measuring the mass transfer coefficient and relative porosity of the collagen coated PTFE membrane for [^{14}C]-sucrose, [^{3}H]-vinblastine and [^{14}C]-Platinum compounds

Transport of radiolabelled compounds through the membrane was measured in the absence of the MCL.

3.11. Synchrotron resonance induced X-ray emission (SRIXE) of MCL cross sections

MCLs were harvested at the end of the transport experiment with the Pt(IV) compound and after routine histological processing 20 μm sections were cut from the paraffin blocks. The sections were mounted on Formvar coated plastic specimen holders. Micro-SRIXE experiments were performed on beamline ID22 at the European Synchrotron Radiation Facility (ESRF), Grenoble, France. Data analysis of SRIXE imaging was performed by choosing regions of interest (ROIs) within each scan. Such ROIs typically included one ROI encompassing the majority of the scan area ("whole MCL"), another encompassing the free surface of the tissue (*i.e.* DC surface), and another encompassing part of the PTFE membrane (*i.e.* RC surface). The Pt-Lα fluorescence line was used to quantify platinum content. Elemental content of ROIs were compared to that of the "whole MCL" ROI, and the zinc content of the ROI.

3.12. Data analysis

Data presented are mean ± SD or mean ± SEM. Data were analysed with ANOVA or two-tailed Student's t-test using GraphPad Prism 3.2 software and $p < 0.05$ was considered statistically significant. Diffusivity and the rate of cellular uptake were determined using programs written in Matlab 7.0.1 software based on the mathematical models described in the *Appendix*.

4. Results

4.1. Characterisation of MCL

4.1.1. Growth and Histology of MCL

The thickness of the PTFE membranes was measured on cross sections using an eyepiece graticule on an upright light microscope and it was 38 ± 1 μm. A minimum of 3 x 10^5 DLD1 cells were required to fully cover the PTFE membrane as a confluent monolayer. The MCL grew from 24.3 ± 5.7 μm on day 2 to a thickness of 172 ± 9 μm by the 14^{th} day. MCLs harvested on the 7^{th} day (131± 10 μm thickness) were used in subsequent transport experiments. The MCL generated by DLD1 cells displayed much of the cellular heterogeneity found in solid tumours. The cells close to the PTFE membrane had large cigar shaped nuclei, while those towards the "upper" surface gradually became flattened. The distribution of proliferating cells also displayed temporal and spatial heterogeneity in MCL. At later stages of the growth curve, the non-proliferating cell mass appeared in the central region of the MCL. The MCL were hypoxic (detected by pimonidazole) towards the upper surface of the tissue. This suggests that the MCL model is a good representation of solid tumour architecture and the associated microenvironment.

4.1.2. mdr1 mRNA and P-gp expression in MCL

The *mdr1* mRNA levels of DLD1 cells were compared to cells known to express low ($MCF7^{WT}$), intermediate (Caco2) and high (NCI/ADR^{Res}) levels of P-gp. DLD1 cells expressed levels of *mdr1* mRNA intermediate between the Caco2 and NCI/ADR^{Res} cell lines. This mRNA expression resulted in measurable protein expression in DLD1 cells cultured as monolayers and as MCL.

4.1.3. Correlation between MCL thickness and [^{14}C]-sucrose flux

The flux of [^{14}C]-sucrose through the PTFE membrane only was faster than in the presence of MCL and in general the [^{14}C]-sucrose flux was slower through thicker MCL. However, the correlation between [^{14}C]-sucrose flux and the thickness of the MCL was weak and the age of the culture predicted the thickness of the MCL more accurately.

4.2. Drug transport assays

4.2.1. Mass transfer coefficient (MTC) and relative porosity of the membrane

The flux of radiolabelled compounds between the two compartments is hindered not only by MCL but by the PTFE membrane too. In order to quantify the membrane's relative porosity, the diffusivity of the radiolabelled compounds in the bathing medium must be ascertained. The diffusivity of sucrose in aqueous solution at 25°C has previously been measured ($5.228*10^{-6}$ cm^2s^{-1}, [43]) and this value was extrapolated to 37°C using the Stokes-Einstein equation (7.0×10^{-6} cm^2s^{-1}). The diffusivity of vinblastine (3.28×10^{-6} cm^2s^{-1}) was estimated from its molecular radius using the same equation. NSB of [^{14}C]-sucrose and [^{3}H]-vinblastine to the polystyrene wall of the RC was determined at concentrations used in the transport assay. After two hours 10.9 ± 1.3 % (n = 3) and 31.2 ± 1.7 % (n =3) of the administered [^{14}C]-sucrose and [^{3}H]-vinblastine were bound, respectively. The measured amount of the radiolabelled compound in the RC was corrected with the NSB for all estimations of drug flux through the MCL. The appearance of the two labelled compounds in the RC was measured (C_R) over a 30 min period. Applying the mathematical model described in the *Appendix 7.1*. gave values of $14 \pm 0.3 \times 10^{-5}$ cm s^{-1} as the mass transfer coefficient for sucrose and $12 \pm 0.3 \times 10^{-5}$ cm s^{-1} for vinblastine. The relative porosity of the collagen coated PTFE membrane was almost two fold higher for [^{3}H]-vinblastine ($14 \pm 4 \times 10^{-2}$), than for [^{14}C]-sucrose ($8 \pm 2 \times 10^{-2}$) ($p < 0.05$).

4.2.2. Diffusive transport of [^{14}C]-sucrose and [^{3}H]-vinblastine in the MCL

Flux of sucrose through the MCL is exclusively through extracellular diffusion, since (i) disaccharides cannot be taken up directly by the cells and (ii) colon cancer cells do not express the brush border enzyme sucrase-isomaltase that is responsible for its digestion in intestinal mucosa. [^{14}C]-sucrose flux was used as an indicator of interstitial diffusion and its diffusion coefficient in the MCL interstitium was calculated to be $4.2 \pm 0.9 \times 10^{-8}$ cm^2 s^{-1}. Similarly to sucrose, the flux rate of vinblastine through the MCL was significantly slower than that observed through the PTFE membrane alone. Vinblastine is lipophilic and its distribution was expected to include the intracellular space in MCL. Surprisingly, the mathematical model that was based purely on diffusion, *i.e.* without any reaction term, described the flux of vinblastine through MCL accurately. On the other hand, the measured radioactivity in the RC can represent both vinblastine and its metabolites and thus it can overestimate the flux of the parental compound. However, there wasn't any Cyp3A4, which is

the major metabolising enzyme of vinblastine *in vivo,* detected in the MCL with western immunoblotting techniques (data not shown). The measured diffusivity of [^{3}H]-vinblastine through the DLD1 MCL was 1.9 ± 0.2 x 10^{-8} cm^2 s^{-1}. The diffusivities of both compounds decreased approximately 170 fold in the MCL compared to the medium, so the MCL hindered the diffusion of vinblastine to the same extent as it did for sucrose.

4.2.3. Diffusivity and cellular uptake rate of [^{14}C]-Pt(II) and [^{14}C]-Pt(IV) compounds in MCL

Initially, an equivalent fluid level was maintained to avoid hydrostatic pressure driven convective transport. The non-specific binding of the compounds to the plastic surfaces in DC and RC was determined to improve the accuracy of the subsequent quantitative analyses. The cumulative concentration of [^{14}C]-Pt(II) and [^{14}C]-Pt(IV) were expressed as a percentage of the initial drug concentration in the DC. The rate and extent of flux was greater for both drugs in the absence of the MCL and there was no difference between [^{14}C]-Pt(IV) and [^{14}C]-Pt(II) with respect to their cumulative appearance in the donor compartment. Mathematical modelling without a cellular uptake parameter did not fit the data obtained in the presence of an MCL, but was an accurate reflection for passage across the PTFE membrane alone. The mathematical model comprising an uptake coefficient was used to quantify flux of the compounds through the solid tumour model. The diffusion coefficients (D_c) of platinum compounds in solution ($8.2*10^{-6}$ cm^2s^{-1} for both compounds) were calculated according to the Stokes-Einstein equation using the molecular radius (MR). The mass transfer coefficients (k) and relative porosity (ψ) values of the two platinum compounds through MCLs were indistinguishable (MTC: $2.7\pm0.6*10^{-5}$ cms^{-1} vs $2.5\pm0.4*10^{-5}$ cms^{-1}, ψ: $1.2\pm0.3*10^{-2}$ vs $1.2\pm0.2*10^{-2}$). The coefficient of diffusion was ascertained for the Pt(IV) and Pt(II) drugs through the MCL model utilising the parameters of mass transfer coefficient (k), MCL thickness (x_M), diffusivity in the medium (D_1) and initial drug concentration (C_D). The calculated diffusion coefficients (D_M: $17.5\pm2.6*10^{-8}$ cm^2s^{-1} vs $17.8\pm3.1*10^{-8}$ cm^2s^{-1}) and cellular uptake (g: $17.7\pm5.5*10^{-2}$ min^{-1} vs $17.7\pm5.5*10^{-2}$ min^{-1}) rates were not different for the two platinum compounds, reflecting similar flux parameters for the two classes of platinum complex in a solid tissue.

4.2.4. Cellular accumulation of [^{14}C]-Pt in the MCL; SRIXE Analysis

Accumulation of Pt compounds in the MCL was determined by measuring the radioactivity retained in the PTFE membrane ± MCL after the transport assays. The radioactivity

associated with the membrane was higher in the presence of the MCL, although there was no significant difference between [^{14}C]-Pt(II) compared to [^{14}C]-Pt(IV). Data from the mathematical modelling above suggested a significant degree of uptake for Pt-drugs within the MCL tissue. Image analysis using SRIXE was used to confirm this. The data shows that tissue that had been exposed to the [^{14}C]-Pt(IV) drug was associated with an incorporated Pt-signal. This confirmed the experimental and mathematical modelling data and demonstrates that flux through solid tissue is associated with a non-negligible degree of Pt-drug uptake into cells. Quantitation of the Pt signal across the tissue revealed a greater Pt(IV)-complex concentration at the upper surface (*i.e.* facing the DC).

4.2.5. The effect of hydrostatic pressure on the flux of [^{14}C]-Pt(IV) through MCL

One proposed way of improving penetration is to increase the hydrostatic pressure to counteract the increased interstitial pressure generated in tumours. The MCL system provides a convenient means to investigate whether hydrostatic pressure can modulate the flux of [^{14}C]-Pt(IV) through solid tumour tissue. Hydrostatic pressure may be varied by simply altering the fluid levels in the DC and RC and was applied in both the same and opposite directions to the concentration gradient of the MCL system. At a hydrostatic pressure of -3 mm H_2O (*i.e.* against concentration gradient) the amount of penetration was reduced from 0.82±0.01% to 0.73±0.01%. In contrast, as the hydrostatic pressure was increased to +4 mm H_2O (*i.e.* along the concentration gradient), the flux of [^{14}C]-Pt(IV) was increased to 1.06±0.08%. The data therefore indicates that even a relatively minor hydrostatic pressure gradient was capable of a significant influence on the flux of [^{14}C]-Pt(IV) through the MCL tumour model.

4.3. Diffusion of vinblastine in a cylindrical model system of colon cancer tissue

Vinblastine is administered IV to patients and it is delivered by the blood to the cancer tissue. Blood vessels can be considered as tubes and drug enters tissues in all radial directions from the vessels. To mimic this situation we designed a model system with radial symmetry from blood vessels running parallel to each other and assuming that the surrounding space was filled with colon cancer tissue with the same cell volume fraction and impedance as in the MCL. The distance between two vessels represents the average intercapillary distance in tumours. The tissue distribution of vinblastine was estimated after a single dose of the drug with blood concentration decreasing according to a four compartment model. In this model

the 70 μm ring of cancer tissue proximal to the vessel wall will experience the highest exposure to vinblastine, while tissues further away will be relatively spared.

5. Discussion

5.1. The MCL as an experimental model system

In this study we aimed to provide a model for investigating kinetics of chemotherapeutic drug flux in solid tumour nodules. MCL was reproducibly cultured from DLD1 colon cancer cells. The MCL system is widely accepted as a model of non-vascularised regions of solid tumours. The experimental model coupled with mathematical analysis proved suitable for measuring the diffusivity of chemotherapeutics in cancer tissue. The MCL grown from DLD1 colon cancer cells recapture many key features of colon adenocarcinoma, *e.g.* cellular morphology and severe dysplasia. Furthermore, MCLs display heterogeneity of cell cycle status (*i.e.* proliferating and quiescent) in addition to having a high cell density and localised regions of hypoxia, which are common features of many solid tumours. The applied mathematical model requires that the MCL retain their architecture and cell viability during the experiment. The duration of the flux assay was significantly shorter than the doubling time of DLD1 cells in monolayer cultures (34 hours, data not shown) and the estimated time required by a cell to undergo apoptosis (12-24 hours). Hence, we can assume that there was neither significant proliferation nor cell loss during the experiment. In order to exclusively measure diffusion, the experimental conditions should exclude convection due to a temperature gradient or hydrostatic pressure. To ensure this, the experiments were conducted in an incubator and the fluids in the donor and receiver compartments were kept at the same level, unless otherwise specified. This experimental set-up was validated by measuring the flux of [^{14}C]-sucrose, which is neither taken up nor metabolised in the MCL. The mathematical model based on Fickian diffusion was able to describe the sucrose flux through the MCL. Thus, the presented transport assay is suitable to measure the diffusivity of various compounds through the extracellular space of the MCL and deviations of the flux from the diffusion based mathematical model could signal the interaction of the drug with the tumour tissue.

5.2. Transport of the ABCB1 substrate vinblastine

The flux of vinblastine through MCL was compatible with extracellular diffusion. The tissue distribution profile based on the diffusivity of vinblastine predicted that cells up to 70 μm away from the circulation will get the highest exposure to the drug. The flux of vinblastine

through the MCL was accurately described by the Fickian diffusion model suggesting that vinblastine was not metabolised or taken up by cells in this system. Vinblastine, which is a lipophilic compound, was expected to enter cells and that its flux through the MCL will deviate from Fickian diffusion. However, vinblastine is also a substrate of multidrug ABC transporters, which are expressed in colon cancer cells. We've found that P-gp is expressed at levels intermediate between parental and drug selected cell lines and P-gp in DLD1 MCL may have restricted vinblastine distribution to the extracellular space. This is the first report that provides a quantitative measure for the actual diffusion coefficient of vinblastine in 3-D tumour tissue models. To date there are only a few data available about drug diffusivity in tumor tissue models and our results are in the same range that has been reported previously. The penetration of vinblastine through MCL has been reported previously. However, kinetic parameters of penetration such as $T_{1/2}$ are not constants and therefore dependent on specific experimental conditions. Furthermore, penetration depends not only on the diffusivity of a compound, but also on the hydraulic conductance of the tissue if there is any convection. In addition, penetration can be further diminished by extensive cellular uptake, metabolism or binding to specific receptors. We have demonstrated that the diffusion of vinblastine was app. 170 times slower in tumour tissue than in solution and 2.5 fold slower than that of sucrose. Which components of the extracellular space in the MCL play a role in limiting vinblastine diffusion? The presence of extracellular matrix in 3-D tumour models could increase the exerted hindrance (i.e. impedance) of the tissue and should be proportional to the size of molecules. However, experimental results do not support a strict correlation between molecular size and diffusivity in tissues. Similarly, in this study the impedance of the MCL was equal for sucrose and vinblastine, despite the fact that their respective molecular radii are different. It is possible that the geometry of the extracellular space is responsible for slowing down diffusion of solutes in tissues. To further specify, based on mathematical models Tao and Nicholson concluded that impedance is not dependent on cell shape but on the relative volume of the extracellular space. These findings emphasise the role of tight cell packing and intercellular contacts as limiting factors of transport in the interstitium. Thus, it is necessary to dissect flux data, as flux through the 3D model membrane is a sum of many different processes. Would it be beneficial to somehow increase the flux of vinblastine in tumour tissues? Vinblastine penetration into tissues with high interstitial pressure will be poor and it will likely to reach cells only in the vicinity of blood vessels. Previously the efficacy of vincristine was correlated with the proliferating population of cells in monolayers, tumour cell spheroids (TS) and tumour xenografts. Furthermore, we have shown that quiescent cells are

insensitive to vinblastine in a modified TS model. The mitotically active cells in solid tumours reside in close proximity to capillaries and even slowly diffusing vinblastine can penetrate to sufficient depth to reach those cells. On the other hand, cells further away from the circulation are not sensitive to vinblastine, hence increased penetration of vinblastine is unlikely to improve therapeutic efficacy. Drug distribution and target cell populations should ideally overlap in tumours and the MCL model can be used to identify drugs that fall short of this requirement.

5.3. Transport of platinum compounds

Platinum based chemotherapy remains a vital component of many treatment strategies in oncology. As with the majority of "genotoxic" anticancer drugs, the Pt-complexes are beset by problems with toxicity, resistance and poor pharmacokinetic properties. A great deal of effort has been placed in generating more potent and selective derivatives of conventional Pt(II) drugs such as cisplatin. In particular, the six coordinate Pt(IV) complexes offer several advantages including lower reactivity and a greater potential for introducing hydrophobicity to facilitate cellular uptake. There is little information available on either class of Pt-drug relating to their behaviour in solid tumour tissue, so the MCL as solid tumour model was utilised to quantify flux behaviour of Pt(II) and Pt(IV)-complexes. Flux through the solid tumour model was associated with significant cellular accumulation and was sensitive to changes in the applied hydrostatic pressure. Both of the compounds tested were able to completely penetrate the MCL tissue with the diffusion coefficients of $17.5 \pm 2.6*10^{-8}$ cm^2s^{-1} for the Pt(II) and $17.8 \pm 3.1*10^{-8}$ cm^2s^{-1} for the Pt(IV)-complex. These rates of diffusion were almost ten fold higher than that for the hydrophobic anticancer drug vinblastine ($1.9 \pm 0.2*10^{-8}$ cm^2s^{-1}) and four fold higher than of the highly hydrophilic sucrose ($4.2 \pm 0.9*10^{-8}$ cm^2s^{-1}). A similar rapid flux (*i.e.* % drug min^{-1}) of cisplatin compared to vinblastine has been reported in MCL comprising bladder cancer cells, whilst etoposide, gemcitabine and paclitaxel displayed faster rates of flux. A major advantage of the current approach is the ability to estimate the actual diffusion coefficients, which may be directly applied to the prediction of tissue drug concentrations; for example, as a function of the distance from a blood vessel. This rapid flux across the MCL should be considered a positive feature for such compounds. A considerable emphasis in the design of platinum drugs has been placed on developing lipophilic drugs that can assist in circumventing diminished cisplatin accumulation in resistant cell lines, however it may be that defeating unicellular resistance may come at the expense of tissue penetration, and therefore create the problem of multicellular resistance. It seems that hydrophilic drugs

such as those examined here allow for excellent tissue penetration. If this feature can be coupled with a more readily reducible Pt(IV) compound (the Pt(IV) compound studied here is the most inert in a series studied), a drug with good penetration and cytotoxic potential may be arrived at. The idea of improving cell penetration and nuclear targeting at the same time by adding an anthraquinone moiety to the Pt(II) backbone (Pt-1C3) was explored. Pt-1C3 and 1C3 alone had similar IC_{50}'s and were more potent than cisplatin in citotoxicity assays with DLD1 cells. However, confocal microscopy revealed that Pt-1C3 was sequestered in the lysosomes and spared the nucleus. Similar discouraging results were published about the dinuclear anthraquinone modified Pt drug tested on drug resistant A2780 cells. The Pt(IV) compound used in the present study has been shown to display lower cellular accumulation in well oxygenated monolayers of cancer cells and lower serum protein binding compared to the Pt(II) parental derivative. Therefore Pt(IV) would be expected to display higher flux rates across MCL than Pt(II); unless the compound is rapidly converted to the parental Pt(II) version. The latter is highly unlikely since even following a 24 hour incubation period in cancer cells detection with micro-XANES revealed that the Pt(IV)-complex was not significantly reduced to the four coordinate form. The two Pt compounds had undistinguishable flux kinetics across the MCL and in both cases the mathematical model required the inclusion of a specific component to account for cellular uptake within the tissue. Similarly, the calculated diffusion constants and cellular uptake rates also failed to detect any pharmacokinetic differences between the two compounds. What is the explanation for the similar rates of flux through the solid tissue given the physico-chemical differences between Pt(II) and Pt(IV) species? Perhaps despite the increased propensity to cross membranes, the accumulation of [^{14}C]-*cis,trans*-[$PtCl_2(OH)_2$(en)] is limited by the lack of reactivity. For example, if the latter was more reactive and thus converted to the corresponding Pt(II) compound the concentration gradient into the cell for the Pt(IV) would remain high enough to ensure greater uptake. Consequently, future Pt(IV) development would require taking into account both transbilayer diffusion and the intracellular reactivity. In addition to allowing detailed flux analysis, the mathematical model predicts that a concentration gradient would exist within the MCL decreasing from the free surface of the tissue towards the semiporous PTFE membrane (*i.e.* DC→RC direction). The SRIXE element array qualitatively demonstrated that the distribution of the Pt(IV) compound was similar to that predicted by the mathematical model, *i.e.* more Pt was detected close to the free surface of the MCL. This is the first report, where the distribution of drugs within the MCL was experimentally verified and compared to the one predicted by mathematical modelling. That more Pt was observed

close to the upper surface of the MCL is in keeping with previous observations with cisplatin detected by antibody in squamous carcinoma cell spheroids, and a Pt-porphyrin compound in J82 spheroids observed by fluorescence microscopy that inferred more intense drug signal (though both relied on a molecular signature other than the active Pt centre itself) at the periphery of spheroids. In the case of the Pt-porphyrin complex, it took 24 hours before uniform drug distribution could be achieved, though this is not reflective of the drug distribution expected to be achieved by a pulse of chemotherapeutic agent. SRIXE assay enabled direct quantification of Pt(II) and Pt(IV) distribution within tumour cell spheroids. Both compounds showed similar distribution, *i.e.* being more abundant at the periphery of the spheroid and less so in the middle region. These results are in line with results from MCL experiments showing a steep downwards gradient of Pt content going from the surface to the middle of the MCL. Most solid tumours have high interstitial pressures that slow drug transit and filtration through tissue and consequently drug movement in cancer tissue is largely limited to diffusion. Restoring the physiological filtration by increasing the hydrostatic pressure is a possible option to improve drug delivery to deeper layers of cancer cells within the 3-dimensional solid tumour mass. Our results show that since the flux of the two compounds was sensitive to hydrostatic pressure that convection could potentially increase the penetration of tumour mass by platinum drugs. This principle, known as convection enhanced delivery (CED), has actually been utilised to treat intracranial tumours where the confined space further increases the effects of interstitial pressure. Cisplatin is expected to be useful via local drug delivery in intracranial tumours due to its reported rapid flux rate, although an enhancement by CED has not yet been examined.The data from the present investigation provides hitherto unknown pharmacokinetic properties for two platinum compounds; namely the absolute diffusion coefficient through solid tumour tissue. Moreover, both experimental and mathematical models provide a convenient means to facilitate the *in vitro* development of novel six-coordinate platinum compounds; particularly to enable a bridge between observations in simple cell monolayer systems to the complexities associated with poorly vascularised hypoxic tumours *in vivo*.

6. Summary

The three-dimensional organisation of cancer cells in solid tumours contributes to the multi-factorial problem of drug resistance. Impaired diffusion through tumour tissue is often thought as a major contributing factor in resistance, yet elucidating intra-tumoural distribution

of anti-cancer drugs is often neglected. Our approach to these problems was to develop tools that can help to estimate drug distribution in vivo.

I. 3-D tumour models were selected to serve as model systems and drug transport was measured in these models.

II. A mathematical model was developed to describe the distribution of anti-cancer drugs through solid tumour tissue.

III. The flux of various compounds was measured through the multicell layer tumour model comprising DLD1 colon cancer cells. Fluxes were determined for sucrose, vinblastine and [^{14}C]-labelled [$PtCl_2$(en)] and cis,trans-[$PtCl_2(OH)_2$(en)] drugs.

IV. The mathematical model provided the diffusion coefficients for these compounds and together with experimental data it suggested a significant cellular uptake of the platinum compounds in the transport system.

V. Although slow diffusion delays vinblastine penetration into the avascular regions of tumours, the proliferating cells susceptible to mitotic spindle poisons are generally in the perivascular area of tumours.

VI. The flux of the Pt(IV) compound through the MCL was not significantly different to that of the Pt(II)-drug nor were the diffusion coefficient or tissue uptake; the latter confirmed with elemental imaging analysis by synchrotron radiation induced X-ray emission.

VII. The flux of the Pt(IV) through the MCL was increased by hydrostatic pressure, thereby demonstrating the potential to target cancer cells further away from the vessels with six-coordinate platinum drugs.

The experimental and mathematical model described has broad applicability for the study of anticancer drug diffusion in 3-D tumour tissue. The mathematical model enables accurate quantification of drug pharmacokinetic behaviour within solid tissue and may be adapted to incorporate the influence of factors mediating pharmacokinetic drug resistance. Determination of the diffusivity of chemotherapeutic agents can help to identify compounds with potential in pharmacokinetic drug development.

7. Appendix

7.1. Mathematical model to describe drug flux through Transwell-Col membranes only

The experimental system is modelled as a closed system with mass conservation. Furthermore the flux through the membrane equals the change of concentration of molecules in the receiver compartment. The following equation was arrived at and the fitted parameter was the mass transfer coefficient (k):

$$C_R(t) = \frac{V_D C_{D_0}}{V_R + V_D}\left(1 - e^{-\frac{Ak}{V_R}\left(\frac{V_R}{V_D}+1\right)t}\right). \qquad \text{(Equation 1)}$$

7.2. Mathematical model to describe flux of radiolabelled compounds through the MCL and the membrane

The flux of radiolabelled compounds through the MCL was modelled as Fickian diffusion. The concentration of compounds in the donor compartment was kept constant during the experiment. The flux at the interface between the MCL and the membrane was continuous and can be described as

at $x = x_M$ $$-D_M \frac{\partial}{\partial x} C(x_M, t) = k\left(C(x_M, t) - C_R(t)\right). \qquad \text{(Equation 2)}$$

Furthermore the governing equation for the drug concentration in the RC ($C_R(t)$) is

at $x = x_M$ $$\frac{d}{dt} C_R(t) = \frac{Ak}{V_R}\left(C(x_R, t) - C_R(t)\right). \qquad \text{(Equation 3)}$$

Eq. 2 and 3 are coupled partial differential equations, which were used to write a program in Matlab 7.0.1 to simulate the experimental situation to calculate impedance (Γ) and D_M.

7.3. Mathematical modelling of vinblastine distribution in colon cancer tissue with radial symmetry

The model system consists of capillaries running parallel to each other and surrounding colon cancer tissue. The distribution of vinblastine after a single dose from the central vessel into the surrounding cancer tissue was described as:

$$\frac{\partial c}{\partial t} = \frac{1}{r}\frac{\partial}{\partial r}\left(D_M r \frac{\partial c}{\partial r}\right). \qquad r_1 < r < r_2 \qquad \text{(Equation 4)}$$

Equation 4 was solved numerically and tissue drug concentrations were plotted against distance from the centre of the vessel.

7.4. Mathematical Model to Describe Intra-MCL Drug Pharmacokinetics

The transport of radiolabelled platinum compounds through the MCL was using a modified version of the model described in chapter 7.2, *i.e.* included another term to account for cellular uptake. As the concentration only varies along the x axis we can describe the diffusion of the drug in the MCL by

$$\frac{\partial c}{\partial t} = \frac{\partial}{\partial x}\left(D_M \frac{\partial c}{\partial x}\right) - g * \phi * c \,, \qquad \text{(Equation 5)}$$

where g is the rate of cellular uptake of the compound, ϕ is the cell volume fraction and c is the drug concentration.. D_M can be defined by the impedance of MCL and the diffusivity of the compound in medium ($D_{1,}$ cm^2 s^{-1}) as

$$D_M = \Gamma * D_1 \qquad \text{(Equation 6).}$$

The concentration in top compartment is calculated from the mass balance by considering the flux of molecules that enters the MCL, *i.e.*

$$\frac{d}{dt} C_D(t) = \frac{AD}{V_D} \frac{\partial c}{\partial x}\bigg|_{x=0} \text{ with initial condition } C_D(t=0) = C_{D_0}. \qquad \text{(Equation 7)}$$

The continuity of the flux at the interface between the MCL and the membrane gives us another boundary condition

$$\text{at } x = x_M \qquad -D_M \frac{\partial}{\partial x} C(x_M, t) = k\left(C(x_M, t) - C_R(t)\right). \qquad \text{(Equation 8)}$$

Furthermore the governing equation for the drug concentration in the RC (C_R(t)) is

$$\text{at } x = x_M + \Delta x \qquad \frac{d}{dt} C_R(t) = \frac{Ak}{V_R}\left(C(x_R, t) - C_R(t)\right). \qquad \text{(Equation 9)}$$

Eq. 5-9 are coupled partial differential equations, which were used to write a program in Matlab to simulate the experimental situation to calculate Γ, D_M and g.

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