TUMOUR MICROENVIRONMENT

Drug penetration in solid tumours

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Abstract | To be most effective anticancer drugs must penetrate tissue efficiently, reaching all the cancer cells that comprise the target population in a concentration sufficient to exert a therapeutic effect. Most research into the resistance of cancers to chemotherapy has concentrated on molecular mechanisms of resistance, whereas the role of limited drug distribution within tumours has been neglected. We summarize the evidence that indicates that the distribution of many anticancer drugs in tumour tissue is incomplete, and we suggest strategies that might be used either to improve drug penetration through tumour tissue or to select compounds based on their abilities to penetrate tissue, thereby increasing the therapeutic index.

Interstitial fluid pressure
The hydrostatic pressure within the interstitial fluid of solid tissue. In tumours, disordered vasculature and the absence of functioning lymphatics is associated with increased interstitial fluid pressure compared with normal tissues.

Extracellular matrix
The complex group of molecules that exist in tissue outside cells.

Clonogenic cell
A cell with the ability to generate progeny that will form a colony of a predetermined minimum size when placed under appropriate conditions. Such cells might represent tumour stem cells, which can regenerate a tumour after treatment.

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Most of the ~10^{13} cells in the human body are within a few cell diameters of a blood vessel. This remarkable feat of organization facilitates the delivery of oxygen and nutrients to the cells that form the tissues of the body. It also enables the efficient delivery of most medicines. However, the homeostatic regulation of tissue and the growth of blood vessels break down in solid cancers. Tumour cells often have the potential for more rapid proliferation than the cells that form blood capillaries[1,2]. So, the proliferation of tumour cells forces vessels apart, reducing vascular density and creating a population of cells distant (>100μm) from blood vessels[3], a process that is exacerbated by a poorly organized vascular architecture[4,5], irregular blood flow[6–9] and the compression of blood and lymphatic vessels by cancer cells[10]. The resultant hypoxia is recognized as a hallmark of cancer, and several strategies have been developed to target cells that have compromised oxygen status[11]. In addition, the disorganized vascular network and the absence of functional lymphatics[12,13] causes increased interstitial fluid pressure (IFP)[14–16]. Finally, the composition and structure of the extracellular matrix (ECM) can slow down the movement of molecules within the tumour[17–19]. Overall, these characteristics of the tumour microenvironment limit the delivery of anticancer drugs to cells that are situated distal from functioning blood vessels; this delivery problem is the subject of this Review.

If anticancer drugs are unable to access all of the cells within a tumour that are capable of regenerating it (that is, clonogenic cells or tumour stem cells), then whatever their mode of action or potency, their effectiveness will be compromised. Perhaps more importantly, the effectiveness of new molecular medicines to treat cancer will be jeopardized if they cannot efficiently penetrate tumour tissue to reach all of the viable cells[20]. For a cancer treatment to be curative, it must have access to all such tumour cells, as the survival of one cell could form the focus of tumour recurrence. Therefore, large distances between tumour cells and functional blood vessels need only occur rarely in tumours to be vitally important.

As a consequence of the poorly organized vasculature in solid tumours, there is limited delivery of oxygen and other nutrients to cells that are distant from functional blood vessels. Poor vascular organization also leads to the build-up of products of metabolism, such as lactic and carbonic acid, which lower the extracellular pH[21–23]. The presence of hypoxic cells in solid tumours has long been recognized[3], and there is a large body of evidence indicating that hypoxic cells are relatively resistant to radiation treatment and can repopulate the tumour after radiotherapy; that is, there are stem cells in hypoxic regions of some tumours[24–26]. A gradient of decreasing tumour cell proliferation forms with increasing distance from tumour blood vessels[27,28], in parallel with decreasing nutrient and oxygen concentration. There are at least three potential reasons why cells that are distant from blood vessels might be resistant to conventional chemotherapy (FIG. 1): first, most anticancer drugs exert selective toxicity on cycling cells, so that non-proliferating or slowly proliferating cells are resistant[29]; second, some drugs might be less active in hypoxic, acidic or nutrient-deprived microenvironments[5,21]; and third, cells distant from blood vessels might be exposed to low concentrations of drug because of limited drug access, as is reviewed here.

A review of published studies about the resistance of cancers to chemotherapy shows that most address cellular and genetic mechanisms of resistance, whereas

At a glance

- Most studies of tumour resistance to anticancer drugs consider only cellular and/or genetic causes operative at the level of a single cell.
- Many anticancer drugs have limited distribution from blood vessels in solid tumours, which limits their effectiveness.
- Large distances between blood vessels in solid tumours, the composition of the extracellular matrix, cell–cell adhesion, high interstitial fluid pressure, lack of convection, drug metabolism and binding contribute to limited drug distribution.
- New *in vitro* and *in vivo* techniques enable quantitative assessment of drug penetration.
- Several strategies have the potential to improve the distribution of anticancer drugs in tumours, and thereby improve their therapeutic index.
- Drug development strategies where molecules are designed or libraries screened for optimal drug penetration would aid the development of more effective anticancer drugs.

only a small fraction describe the role of the tumour microenvironment. This bias in support of cellular and genetic mechanisms as the dominant influences that affect drug resistance reflects the overwhelming use of monolayer culture as a model in cancer research, a model that does not reflect the microenvironment of solid tumours. Cells in solid tumours are exposed to various microenvironments and to a large gradient of concentration as the drug diffuses from blood vessels. By contrast, cells in monolayer culture are generally exposed to a uniform environment and to a uniform concentration of a drug. The slow accrual of new anticancer drugs has questions about the strategy used to develop them[30,31], which depends on *in vitro* screens that do not model the complex pathophysiology of cancers, including the large intervascular distances that are encountered in many tumours.

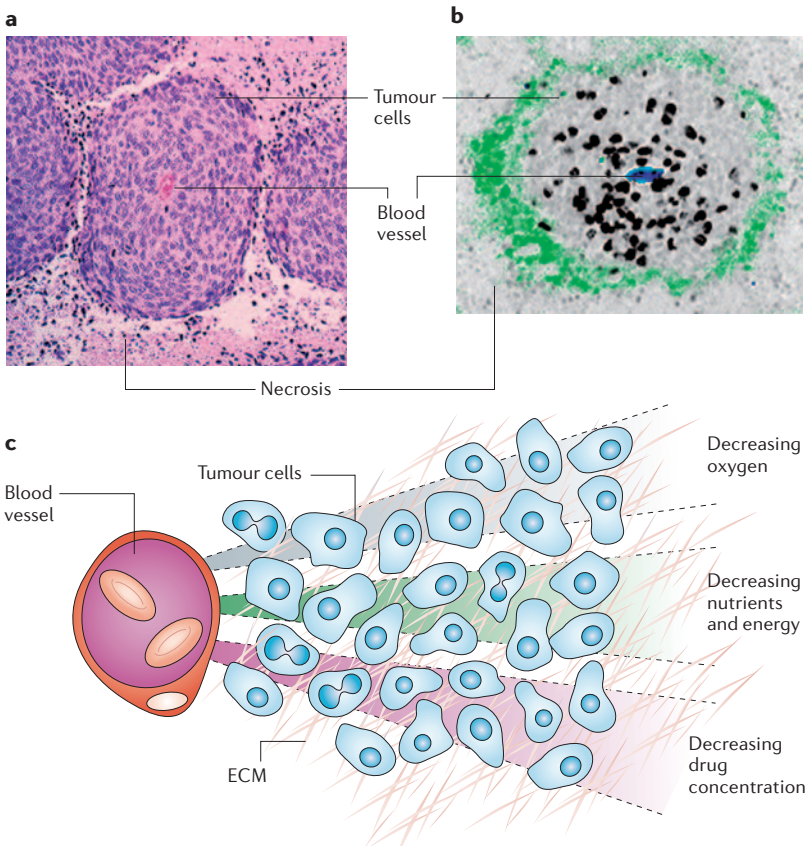

Figure 1 | **Potential reasons why cells distant from blood vessels might be resistant to treatment. a** | Cords of cells surrounding a blood vessel in a xenograft of a cervix cancer. **b** | Cords of cells surrounding a blood vessel in a xenograft of colon cancer. More cells are proliferating (bromodeoxyuridine-labelled black cells) close to the blood vessel. Green staining indicates hypoxic regions identified by pimonidazole. Proliferation occurs primarily close to blood vessels. Endothelial cells are coloured blue. **c** | A diagrammatic representation illustrating the gradients in oxygen, nutrients and energy, and drug concentration.

Properties that influence drug distribution

Before drugs can be introduced into clinical practice their pharmacokinetic properties are studied in animals and in early-phase clinical trials. The mean drug concentration is determined in plasma and in various tissues of the body, including tumours, as a function of time after administration. Drug behaviour is described by parameters such as volume of distribution (V_d), half-lives for disappearance from plasma and clearance from the body[32]. Although these parameters are important for determining overall drug disposition, which might relate to toxicity for certain body organs, and for devising a logical schedule of administration, they give limited information about access to target tumour cells. For example, mitoxantrone has a high V_d because of sequestration within cells caused by DNA binding and entrapment in acid vesicles[33], but has poor penetration into tissue. An anticancer drug might even show an average concentration that is higher in the tumour than in normal tissues, but if tissue penetration is poor only cells close to blood vessels will be exposed to an effective concentration.

As anticancer drugs distribute within tumours they form gradients from tumour blood vessels that change with time as the drug is cleared from the body. The permeability of vessel walls influences drug penetration, but is thought to be insignificant in many tumours where blood-vessel fenestrations have been observed[13,34–36]. Drugs penetrate normal tissues by both diffusion and convection, with a net flow of fluid from blood vessels balanced by resorption into lymphatics. However, tumours often lack functional lymphatics[12,13], which can lead to increased levels of IFP in tumours[14–16], which in turn is likely to reduce convection and thereby inhibit the distribution of macromolecules[20,37].

The physicochemical properties of drugs (for example, molecular weight, shape, charge and aqueous solubility) determine the rate of diffusion through tissue. The penetration of a drug is also dependent on its consumption, which functions to remove free drug, thereby inhibiting further permeation[38] (FIG. 2). Water-soluble drugs distribute most readily in the ECM, and therefore efficiently diffuse around and between cells. Lipid-soluble drugs penetrate lipid membranes, and so can be transported through cells. Consumption includes the effects of metabolism, binding to tissue elements (both specific to the target receptor and nonspecific), and uptake and retention in tumour cells. Retention in tumour cells can be due to binding

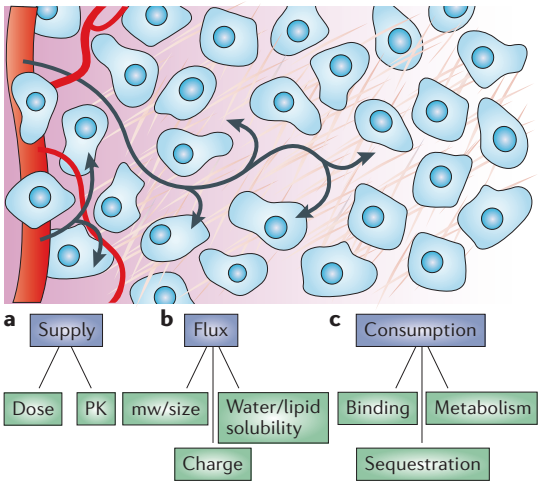

Figure 2 | **Drug distribution in tissue. a** | Supply. The supply of a drug to the tissue will depend on its dose and pharmacokinetics (PK). **b** | Flux. After leaving the vasculature, flux through tissue can occur through extracellular or trans-cellular pathways, depending on relative solubility in water and lipids. Although diffusion through water will vary with size or molecular weight (mw), movement will be hindered by interactions with extracellular and cellular components, and the barrier posed by the cell membrane. **c** | Consumption. Tissue penetration will be determined by the balance between delivery (supply and flux) and consumption. Cellular metabolism will reduce drug penetration and build-up within the tissue, and binding and sequestration can increase net tissue levels of a drug but limit its penetration.

Tumour stem cell
A tumour cell that has the capacity to regenerate the tumour after treatment. Such cells must be eradicated if tumour therapy is to be curative. Stem cells in some tumours can have surface markers that facilitate their identification.

Pharmacokinetics
The time course of drug absorption, distribution, metabolism and excretion within the body.

Volume of distribution
A hypothetical volume calculated by extrapolating the plasma concentration–time curve back to time zero, which represents the volume at which the administered drug seems to have been distributed. Large volumes of distribution imply efficient extravasation, but not necessarily complete distribution in tumour tissue.

Spheroids
Spherical aggregates of tumour cells, grown in tissue culture, which reflect many of the properties of solid tumours. Spheroids have been used for studying the penetration of anticancer drugs into tumour tissue.

Cell contact effect
The growth of tumour cells that are in contact with each other leads to changes in the expression of some genes, and has been shown to influence cellular properties, including response to therapeutic agents, compared with single cells in culture.

at the site of lethal activity (usually DNA), although basic drugs can be sequestered in acidic organelles such as perinuclear endosomes[39,40]. For example, the poor penetration through tissue of doxorubicin (see below) is due to avid binding to DNA and sequestration in acidic endosomes of cells that are proximal to the vasculature.

Experimental drugs have been developed to target hypoxic cells in tumours that are resistant to radiation; they also have considerable potential to increase the effects of conventional chemotherapy, to which hypoxic cells are often resistant. Most of these agents (tirapazamine, AQ4N and PR-104) are pro-drugs, which are reduced under hypoxic conditions to an active form that is cytotoxic. These agents must be able to penetrate tissue efficiently to reach the target hypoxic cells, but as these molecules diffuse from the well-oxygenated cells that surround a blood vessel, the decreasing pO_2 results in increasing metabolism. There is therefore a balance between metabolism (a form of consumption) and efficient penetration. Too much metabolism and no drug will remain to diffuse; too little metabolism of the pro-drug and hypoxic cells will not be killed.

Although physicochemical parameters influence the ability of a drug to penetrate the extravascular compartment of tumours, accurately modelling the many relevant factors is so complex that the measurement of drug distribution using model systems is a more practical method of determining how efficiently a drug penetrates tissue.

Methods for studying drug penetration

In vitro multicellular models have become the most commonly used tools to qualitatively and quantitatively assess drug penetration. Although direct *in vivo* assessment, when feasible, has the advantage of duplicating the clinical environment most closely, *in vitro* techniques offer the advantage of being able to examine the distribution of drugs in the absence of complicating factors such as pharmacokinetics and hepatic metabolism, which often differ between mice and humans. This ability to evaluate drug penetration should give such models an important role in drug discovery and development. Features of solid cancers that are not modelled by multicellular models are variable IFP, the influence of convection[20] (which commonly occurs in the periphery of tumours and might have particular significance for large molecular weight therapeutics) and the influence of stromal cells[18].

Multicellular spheroids. Multicellular spheroids are spherical aggregates of tumour cells that are usually grown in spinner culture, and which reflect many of the properties of solid tumours, including the development of an ECM, tight junctions between epithelial cells and gradients of nutrient concentration and cell proliferation from the exterior to the centre[41,42] (FIG. 3a). Spheroids can grow to a diameter of about 1mm; larger spheroids develop central necrosis and regions of hypoxia[43]. By incubating spheroids in a medium that contains an anticancer drug it is possible to examine the kinetics of drug penetration in histological sections. Doxorubicin and other anthracyclines are recognized by their fluorescence[44–47], and the distribution of radio-labelled drugs has been characterized by autoradiography[48–51]. Although the geometry of drug penetration inwards toward the centre of the spheroid is often not reflective of the *in vivo* situation, spheroids have been very useful in characterizing the distribution of several anticancer drugs and macromolecules such as antibodies[52–54].

Techniques have also been developed that enable the viability of cells to be assessed at different depths in spheroids after their exposure to an anticancer drug. Spheroids can be exposed to trypsin for sequential periods to remove successive layers of cells, and the viability of these cells can be assessed using a colony-forming assay[55]. Alternatively, spheroids can be exposed to the fluorescent dye Hoechst 33342, which itself establishes a concentration gradient from the outside to the centre of the spheroid. Hoechst 33342 binds to DNA, but is minimally toxic so that cells at different depths in spheroids can be separated and sorted by flow cytometry, based on their Hoechst 33342-derived fluorescence, and then evaluated for survival using a colony-forming assay[56,57]. The response of spheroids to drugs differs from that of cells in monolayer culture not only because of limited drug penetration and differences in hypoxia and proliferation status, but also because of the cell contact effect. The growth of tumour cells in

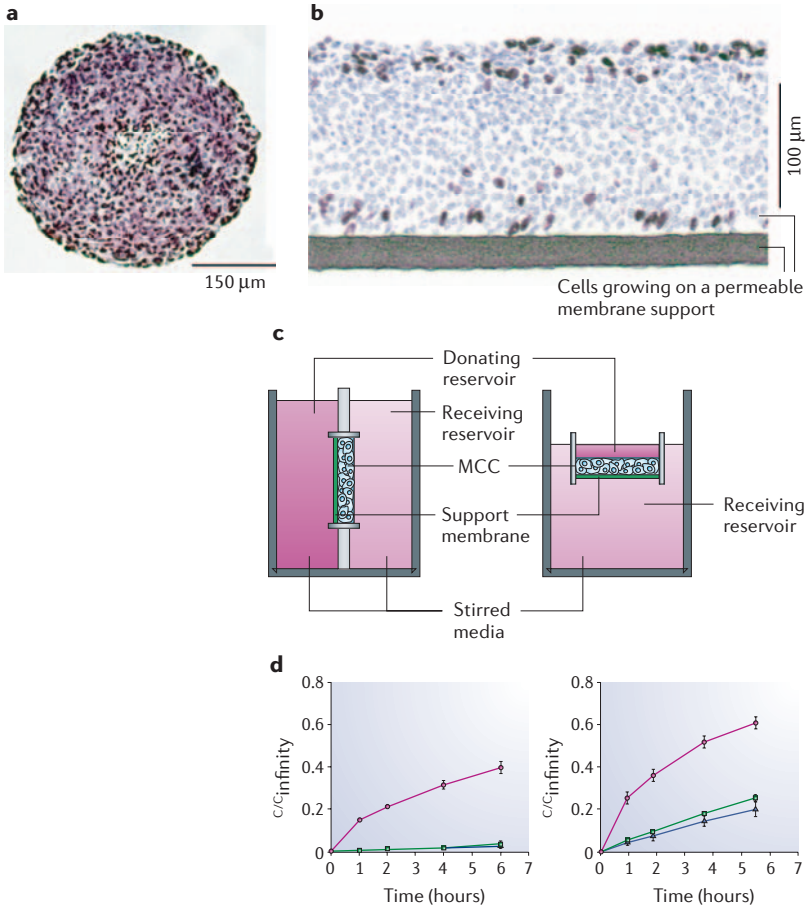

Figure 3 | **Multicellular models and their use to study drug penetration.** **a** | Multicellular spheroids are grown in spinner culture to form spherical aggregates. **b** | Multilayered cell cultures (MCCs) are grown on permeable support membranes; proliferating cells are located predominantly close to the upper and lower surfaces. **c** | Studies of drug penetration are usually performed by separating two reservoirs with an MCC (left) or by floating an MCC on a medium with the drug of interest added to the small compartment above the MCC, and sampling from the lower receiving reservoir (right). **d** | The penetration of a drug through an MCC (lower curves) is usually compared to penetration through the support membrane alone (upper curves), as shown for doxorubicin (left), and 5-fluorouracil (right).

contact with each other leads to changes in the expression of some genes, and has been shown to influence the response to therapeutic agents compared with single cells in culture[58,59]. This feature is common to other multicellular cultures and to tumours *in vivo*[60,61].

Multilayered cell cultures. Multilayered cell cultures (MCCs), also called multicellular layers (MCLs) and multicellular membranes, are comprised of tumour cells grown on a permeable plastic support membrane to form a disc of tissue; they are typically used when they reach 200–250 µm in thickness (~15–20 cell diameters; FIG. 3b). The technique was pioneered by Wilson and colleagues[62], who floated MCCs in a reservoir of stirred media whilst semi-liquid media was placed in the compartment above them. Drug passage from the semi-liquid media through the MCC to the stirred media below was measured with time to provide diffusion coefficients of selected DNA intercalators[62] (FIG. 3c). An alternative system whereby an MCC separates two stirred compartments of liquid media was developed by one of the authors[63] (FIG. 3c): drugs of interest are added to one reservoir, and transport through the culture is measured by sampling from the reservoirs as a function of time. Like spheroids, MCCs have been shown to reflect many of the properties of solid tumours, including the generation of an ECM, gradients of nutrient concentration and cell proliferation, and regions of hypoxia and necrosis in thicker layers[63–66]. Penetration through MCCs can be studied for any drug for which there is an available assay to quantify its concentration in media; the use of radio-labelled drugs is convenient, but analytical methods such as high-performance liquid chromatography or mass spectrometry can also be used, and have the advantage of being able to distinguish between the parent compound and metabolic products, a feature not possible with spheroids. Penetration through an MCC is usually compared with penetration of the drug through the microporous support membrane alone (FIG. 3d), where unstirred boundary layers reduce the effective porosity. MCCs have been used to quantify the penetration through tumour tissue of many anticancer drugs that are in clinical use[66–69], as well as experimental agents, including those designed to kill hypoxic or nutrient-deprived cells where good tissue penetration is essential for activity[62,65,70–73]. MCCs can also be sectioned and the distribution of fluorescent or radio-labelled drugs can be visualized directly, as with spheroids[74].

A recent modification enables MCCs to be used for the direct evaluation of drug distribution in tissue without the need to visualize the drug. Rather than measuring drug flux through the cultures, one side of the MCC is temporarily closed off during exposure, and drug concentration gradients are allowed to form as they would in a tumour. The technique then exploits the unique property of symmetrical growth patterns on the two sides of the culture, which enables the penetration of the drug to be evaluated on the basis of a comparison of the drug's effect on either side of the culture. Using this simple pharmacodynamic model enables drug penetration to be evaluated without the need for detailed information on drug–cell interactions (such as cellular uptake, metabolism and binding), which is required when using flux data to mathematically predict drug penetration[69].

In vivo methods. Studies of the distribution of injected coloured dyes such as lissamine green in sections of animal tumours were made nearly 50 years ago. The heterogeneous staining prompted the authors to state that the vascular architecture of tumours would "affect attempts at immunotherapy and chemotherapy"[75,76]. Early studies of the distribution of drugs in tissue, such as doxorubicin[77–79] or etoposide[80], either by direct fluorescence or immunohistochemistry, showed considerable heterogeneity, and uptake into tumour tissue was observed to be poor compared with normal tissues. Without information about the location of blood vessels the conclusions regarding

Multilayered cell culture
Also called a multicellular layer or a multicellular membrane, an MCC contains tumour cells that grow on a permeable plastic support membrane to form a disc of tissue. MCCs reflect many of the properties of solid tumours, and are used to study the penetration of anticancer drugs through tumour tissue.

Pharmacodynamics
The effects of a drug on the cells and tissues of the body.

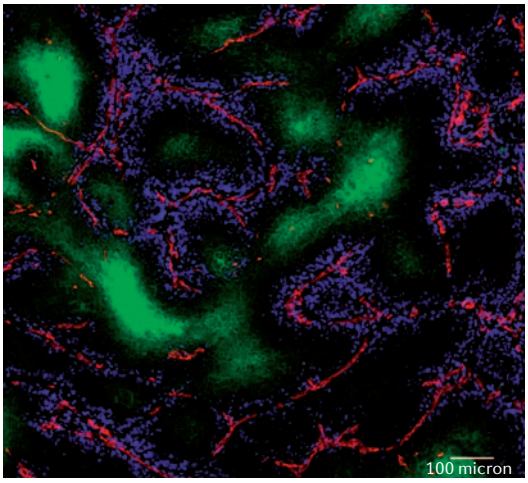

Figure 4 | **Distribution of doxorubicin *in vivo*.** A Section from a mouse mammary tumour showing the distribution of doxorubicin (blue) in relation to tumour blood vessels (red) and regions of hypoxia (green). Note that doxorubicin is distributed around tumour blood vessels. Bar = 100μm. Reproduced with permission from REF. 102.

micro-regional distribution were limited. Despite this, Jain and Simpson-Herren championed the view that variations in drug penetration were responsible for heterogeneity in tumour response[81–83], and suggested that adjuvant chemotherapy might be effective in some patients where very small micro-metastases would be sterilized, whereas in patients with larger metastases limited drug penetration would result in tumour progression.

The technique described of using Hoechst 33342 to establish a concentration gradient in spheroids, followed by fluorescence-activated cell sorting and assessment of cell survival in a colony-forming assay has also been applied to tumours in mice, where the Hoechst dye was injected intravenously following treatment with an anticancer drug[84]. This method showed that doxorubicin activity decreased with an increasing distance from tumour blood vessels, but the technical complexity of the method limits its widespread utility.

Direct assessment of the distribution of drugs in tumour tissue is technically challenging, and requires the quantification of drug exposure to cells at specific locations relative to blood vessels. Window preparations, with which growing tumours are observed directly in the living animal under varying physical conditions or during and after the administration of various agents, including anticancer drugs, have been used successfully to obtain information about temporal changes in the vascular network, gradients of metabolites and diffusion and convection of fluorescent or coloured molecules from tumour vessels into the surrounding tumour tissue[20,37,85–90]. By applying two-photon microscopy, it is possible to image several hundreds of microns deep into tumours[91], and changes in the above parameters have been quantified in response to various treatments[92,93] and have been validated in cancer patients[94].

Other than window preparations, only microscopy applied to tissue sections can currently provide the micro-regional resolution that is required to make quantitative measurements of drug concentration. Unfortunately, most drugs are not amenable to direct measurement because they lack sufficient colour or fluorescent properties (the clinically-used drugs doxorubicin, mitoxantrone and topotecan are exceptions). Autoradiography provides sufficient resolution[95,96], but the complexity of combining autoradiography with immunohistochemical methods to label tumour blood vessels limits the use of this technique. Immunohistochemical labelling of drug adducts, such as cisplatin with DNA, is promising, but has so far been limited by the available antibodies[97,98]. Antibodies are available to bound markers of hypoxia, such as pimonidazole and EF5, and have been used to show that these nitroimidazoles penetrate tissue efficiently[99,100]. Fluorescence-labelled antibodies to human immunoglobulin can also be used to study the distribution in tumour tissue of therapeutic monoclonal antibodies such as cetuximab or trastuzumab by immunohistochemistry.

Fluorescence microscopy has been used to generate distributions of the fluorescent drug doxorubicin in relation to tumour blood vessels, recognized by an antibody to the endothelial cell marker CD31, in cryosections of human and animal tumours[101,102]. Computerized image analysis allows tumour sections to be scanned at high resolution, and thereby enables the fluorescence of doxorubicin to be related to distance from the nearest blood vessel in the section. Hypoxia markers, such as pimonidazole[99] or EF5 (REF. 103), can also be injected to enable the recognition of hypoxic regions using a fluorescent-labelled antibody in the same sections[102] (FIGS 1b and 4). These studies show steep doxorubicin gradients around blood vessels in mouse tumours and human breast cancer[101,102].

An alternative to directly detecting the presence of a drug in tissue is to use fluorescent-labelled antibodies to detect an effect that the drug has on cells. An example of this pharmacodynamic approach is to identify effects on cell proliferation, recognized by antibodies to proliferative markers such as MKI67 or cyclin D1, or following uptake of bromodeoxyuridine (BrdU) into cells during S phase[104,105] (FIG. 5). Similarly, apoptosis can be recognized by antibodies to caspases or other markers of apoptosis. This approach has the advantages that cells exposed to a sufficient concentration of a drug to bring about the cellular effect will be selectively recognized, and that it can be applied to study combinations of agents. However, assessing the effect of drugs on cells that reside distant to blood vessels is complicated by inherent differences in cell sensitivity within the tumour microenvironment, such as those caused by the gradient in proliferation kinetics that occurs as a function of distance from blood vessels[106]. Modelling is required to account for this[106].

Considerable effort is being made to investigate the use of non-invasive imaging techniques such as magnetic resonance imaging (MRI) and positron emission tomography (PET) to evaluate drug distribution in tumours. However, at present the sub-mm resolution of MRI and mm resolution of PET is insufficient to

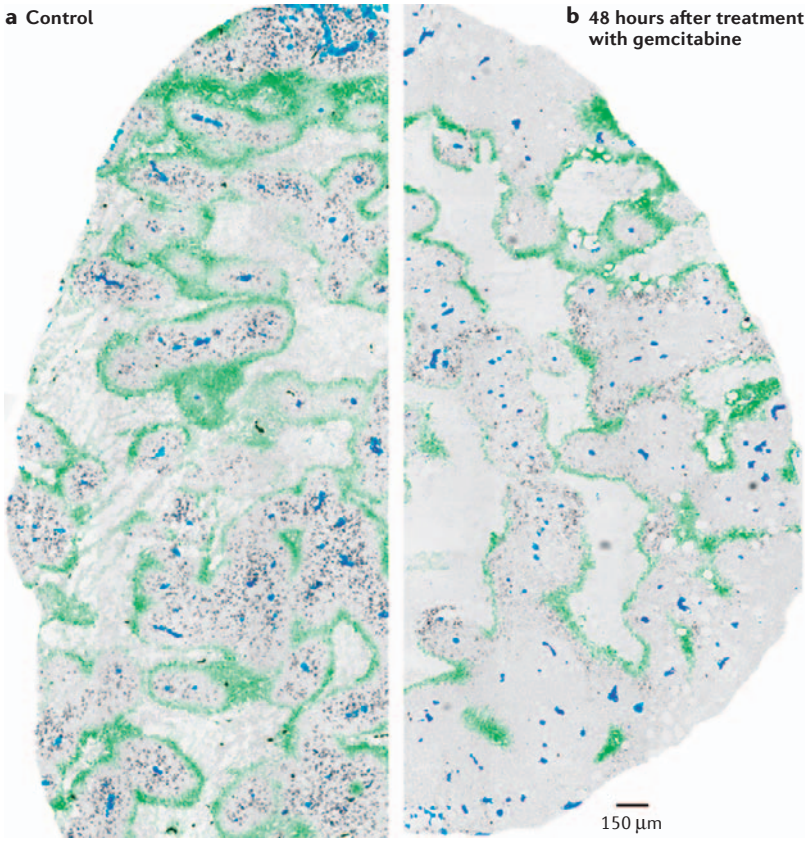

Figure 5 | **Tissue mapping of a human colorectal cancer xenograft.** Sequentially stained and overlaid sections of an HCT116 tumour from untreated (**a**) and gemcitabine-treated (**b**) mice. S-phase cells (black) are distributed around blood vessels (blue) in untreated tumours, but are confined to areas distal to blood vessels and close to hypoxic cells (green) 48 hours after gemcitabine treatment.

detect the microregional variations in drug concentration that occur within tumours[107,108]. Although such techniques could give information about average drug disposition in tumours compared with normal tissues, they will require some 100-fold improvement in resolution before they can be used to study the variation in drug concentration within the microenvironment of tumours.

Distribution of anticancer drugs in tissue

The methods described above have generated substantial information about the penetration of various drugs through tissue, about their distribution in tissue and/or about their activity as a function of depth in tissue. More information is available for doxorubicin than for any other drug, because its fluorescence has facilitated direct detection. Experimental results with doxorubicin are consistent in showing limited penetration into spheroids (and consequent reduced activity against centrally located cells)[44,45,47,54,55,57,109], and this is confirmed in MCCs[67–69] (FIG. 3d) and tumours[101,102] (FIG. 4). Related drugs that also bind avidly to DNA, such as mitoxantrone, also have limited penetration through MCCs[68,69]. Radio-labelled methotrexate and vincristine have also been found to have limited penetration into spheroids[48,49]. The anti-metabolite gemcitabine showed a clear dose-dependent ability to penetrate MCCs[66,104], and caused almost complete cessation of proliferation throughout a tumour *in vivo* at clinical doses. However cells that were distal to tumour blood vessels soon resumed proliferation, indicating that the effectiveness of cell-cycle inhibition was related to the proximity of the cells to tumour blood vessels and, by extrapolation, to drug exposure[104] (FIG. 5).

Given the uniform results, which show limited distribution of doxorubicin in solid tumours, it is legitimate to ask why this drug has been useful for treating solid tumours. Possible explanations include: first, that the efficient killing and removal of proximal cells releases drug that can penetrate to deeper layers of cells and, coupled with the slow clearance of drug from the tissue[49], leads to the effective exposure of cells distal from blood vessels (although it seems probable that rapid clearance from plasma limits the amount of drug that penetrates into tumour tissue); second, sequential cycles of doxorubicin-based chemotherapy lead to the sequential killing of cells at increasing distances from tumour blood vessels, similar to peeling an onion inside-out (although repopulation from surviving distal cells is likely to re-establish tissue architecture between courses of treatment)[110]; third, like many anticancer drugs, doxorubicin is much more toxic to proliferating cells, which are known to be located selectively close to tumour blood vessels[27,28]; and fourth, similar to proliferating tumour cells, stem cells in some tumours can be located close to tumour blood vessels, and could therefore be killed despite limited penetration of the drug. Even if any or all of these explanations are relevant, it is probable that the therapeutic effect of doxorubicin is reduced because of limited penetration, and that improved penetration would increase its efficacy.

The flux through MCCs has been evaluated for agents that are designed to either sensitize hypoxic cells to radiation (such as misonidazole and pimonidazole)[64], or which have direct toxicity to hypoxic cells (such as tirapazamine). In these cases, hypoxic cells located at the diffusion distance of oxygen are the target cells, so it is essential that these drugs penetrate efficiently. Despite compelling preclinical[111] and promising clinical results[112,113], tirapazamine does not seem to penetrate efficiently though tumour tissue[65,71,72,114]. A pharmacodynamic and pharmacokinetic model has shown that the oxygen dependence of metabolism of tirapazamine to its active form enables optimal activity against cells at intermediate oxygen tension[114], and other mechanisms, such as anti-vascular effects, might also contribute to the activity of this drug[100].

An increasing array of molecularly-targeted agents have been shown to have clinical activity, including small-molecule inhibitors of growth-factor receptors such as imatinib, erlotinib and gefitinib, and monoclonal antibodies such as cetuximab and trastuzumab. Some studies have indicated limited penetration of monoclonal antibodies from the surface of spheroids[52], and limited distribution from blood vessels in 2-dimensional window chambers[115]. As yet, there is little information

about the ability of these molecularly-targeted agents to penetrate tumour tissue. Recently, adjuvant trastuzumab has been reported to have the remarkable effect of reducing early recurrences in women with ERBB2 (also known as HER2)-positive breast cancer[116,117] by ~50%, despite the fact that it leads to tumour response in only ~25% of women with advanced metastatic disease[118]. The apparent ability of this monoclonal antibody to sterilize micrometastases might be due to the exposure of the target cells to trastuzumab when the drug is used as adjuvant therapy, compared with restricted drug distribution in large tumours, although its anti-angiogenic activity could also contribute to effects against small metastases[119].

Methods for modifying drug distribution

A better understanding of the factors that determine drug penetration will enable the structural modification of existing drugs or the development of new agents so that they will more effectively penetrate the extravascular compartment. In addition, aspects of the tumour microenvironment that might also be modified to improve drug distribution include cellular biochemistry, blood vessel architecture, haemodynamics and the ECM.

An important opportunity now exists to use these new tools and techniques of assessing tissue penetration and the emerging understanding of extravascular transport to develop agents on the basis of their ability to penetrate tissue. The first examples of drug development efforts based on this approach studied the effect of lipophilicity[120,121], represented by the partition coefficient, and hydrogen bond donors[121] on the diffusion of tirapazamine analogues[120]. A recent report further shows how measurements of extravascular diffusion can improve the ability of a 3-dimensional pharmacokinetic and pharmacodynamic model to predict the activity of tirapazamine analogues against hypoxic cells in tumours[122].

Intermittent or erratic flow through blood vessels is well established in animal tumour models, and gives rise to micro-regions of tumour tissue temporarily deprived of blood flow[6–10,123]. This results in cells, albeit temporally, that reside at long distances from the nearest functioning blood vessel. There is little information as to whether the micro-regional cessation of blood flow also occurs in human tumours, although this seems probable[124]. Some human tumours are known to have increased IFP[14,15], which is also known to occur in animal tumours where there is vascular disorganization and aberrant or absent lymphatics[10,12,16,125,126].

It was expected that inhibiting angiogenesis in tumours would decrease the effectiveness of chemotherapy, because vascular access of anticancer drugs to tumours would be inhibited, and of radiotherapy, because of increasing hypoxia. However, agents that inhibit angiogenesis, such as the monoclonal antibody bevacizumab that is directed against vascular endothelial growth factor (VEGF), seem to enhance the effects of chemotherapy when used to treat some human cancers[127]. Recent experimental work has shed light on this apparent paradox. The administration of the agent DC101, a monoclonal antibody against the VEGF receptor 2 in mice, has been shown to normalize vascular architecture in tumours, improve blood flow to them and reduce IFP[92,93,128]. The proposed mechanism of action includes the 'pruning' of immature vessels and the improvement of the perivascular cell coverage and structure of the basement membrane, which leads to the normalization of the tumour vessels. This effect seems to be temporary, so there might be a window of opportunity after the administration of anti-angiogenic agents when radiotherapy can be given to a better oxygenated tumour, and perhaps when the access of a drug to tumour tissue might be improved[93]. The effect also seems to depend on the tumour model and the anti-angiogenic agents that are used, because other authors have reported a consistent increase in tumour hypoxia after such treatment without a window of improved oxygenation[129,130].

The route that a drug takes as it traverses tissue will depend on its physicochemical properties. The penetration of methotrexate was increased by the co-administration of folate or by reduced pH, both of which decrease cellular uptake of the drug[131]. Similarly, the penetration of doxorubicin was greater through MCCs that were comprised of cells with a high level of expression of P-glycoprotein, and was decreased by inhibitors of P-glycoprotein, which is consistent with increased penetration into tissue when the drug remains in the ECM[132]; this finding was confirmed recently in tumours in animals (Patel, K. and I.F.T, unpublished observations). Indeed, the opposing effects of inhibitors of P-glycoprotein to increase the uptake of substrate drugs into cells close to blood vessels in solid tumours, but to thereby decrease their penetration to more distal cells, might explain in part the disappointing results of clinical trials that have evaluated the concurrent use of chemotherapy and inhibitors of P-glycoprotein[133,134].

The ECM is known to have a rapid rate of turnover, so modification of the ECM therefore has the potential to alter the penetration of anticancer drugs through tumour tissue. The ECM can bind some drugs, and can therefore function as a sink to reduce penetration. The treatment of multicellular spheroids with hyaluronidase increases their sensitivity to chemotherapy agents, although this is specific to certain tumour cell lines[135,136]. The presumed mechanism is an anti-adhesive effect from the breakdown of hyaluronic acid that enables drugs to permeate the extracellular compartment more freely. The penetration of several anticancer drugs was found to be better through MCCs that were derived from an HCT-8 human colon cancer cell sub-line. HCT-8 sub-line cells lack the cellular adhesion molecule α-E catenin, and have a reduced cellular packing density compared with parental cells, which have a higher packing density[74]. High cellular packing density has been associated with increased IFP, and various strategies might be used to reduce both packing density and IFP before other drugs are administered, including anti-adhesive agents (although there would be concern about stimulating metastasis) and pre-treatment with low doses of anticancer agents such as paclitaxel[95,137,138]. In addition, the degradation of fibrillar collagen with bacterial collagenases has been shown to increase the transport of viral vectors in human melanoma xenografts[139].

Partition coefficient
The ratio of the solubility of a drug in two solvents, usually octanol and water. The logarithm of this number (logP) is commonly used to describe relative hydrophobicity or hydrophilicity.

Basic drugs such as doxorubicin and mitoxantrone have been shown to concentrate in perinuclear acidic endosomes[39,140,141], and it might be possible to decrease the proton gradients that drive this sequestration by loading cells with competing weak bases such as chloroquine, as was first shown in MCCs by Hicks and colleagues[62,142], or by inhibiting the ATPase proton pump that establishes the pH gradient in endosomes with drugs such as omeprazole[143]. Recent experiments that have investigated these strategies have shown decreased sequestration of doxorubicin in cells with maintained or increased cellular toxicity, and improved penetration of doxorubicin and mitoxantrone through MCCs[40]. These approaches are being evaluated in mouse tumours by studying the effect of these potential modifiers on distribution of doxorubicin in relation to blood vessels.

Changes in drug formulation can also modify drug penetration in tissue. Potential examples include formulations of doxorubicin encapsulated in liposomes, which effectively alter the pharmacokinetics of the free drug and take advantage of the permeability of tumour vessels to liposomal particles[144], and abraxane, a nanoparticle formulation of paclitaxel bound to albumin that was developed to circumvent the need for cremaphor as a solubilizing agent, but which will also have effects on pharmacokinetics that could lead to improved tissue penetration[145].

Concluding remarks

Advances in adjuvant therapy, where drugs are given to treat residual micro-metastases after primary treatments with surgery and/or radiation, have shown improvements in survival. However, most cancers that recur are ultimately fatal because systemic therapies do not eradicate gross metastatic disease. The tacit assumption that drugs distribute efficiently throughout the tissues of the body does not hold for anticancer drugs. The intervessel distances in tumours can often be large, and can result in some cells receiving sub-therapeutic drug exposure.

Drug development has emphasized screening techniques that are based on the responsiveness of single cancer cells in monolayer culture, and has failed to consider the importance of extravascular drug penetration. The recent development of simple multicellular models, and of quantitative image analysis, could facilitate the measurement of drug penetration as a parameter that could assist in the discovery of drugs that are effective for the treatment of solid tumours.

1. Tannock, I. F. & Hayashi, S. The proliferation of capillary endothelial cells. *Cancer Res.* **32**, 77–82 (1972).
2. Denekamp, J. & Hobson, B. Endothelial-cell proliferation in experimental tumours. *Br. J. Cancer* **46**, 711–20 (1982).
3. Thomlinson, R. H. & Gray, L. H. The histological structure of some human lung cancers and the possible implications for radiotherapy. *Br. J. Cancer* **9**, 539–549 (1955).
This landmark article suggested that the hypoxic cells within human tumours were resistant to radiation therapy.
4. Less, J. L., Skalak, E. M., Sevick, E. M. & Jain, R. K. Micrvascular architecture in a mammary carcinoma: branching patterns and vessel dimensions. *Cancer Res.* **51**, 265–273 (1991).
5. Brown, J. M. & Giaccia, A. J. The unique physiology of solid tumors: opportunities (and problems) for cancer therapy. *Cancer Res.* **58**, 1408–1416 (1998).
6. Intaglietta, M., Myers, R. R., Gross, J. F. & Reinhold, H. S. Dynamics of microvascular flow in implanted mouse mammary tumours. *Bibl. Anat.* **15 Pt 1**, 273–276 (1977).
7. Chaplin, D. J., Olive, P. L. & Durand, R. E. Intermittent blood flow in a murine tumour: Radiobiological effects. *Cancer Res.* **47**, 597–601 (1987).
Describes intermittent blood flow in an experimental tumour and suggests a second mechanism by which tumour cells can become hypoxic.
8. Chaplin, D. J., Trotter, M. J., Durand, R. E., Olive, P. L. & Minchinton, A. I. Evidence for intermittent radiobiological hypoxia in experimental tumour systems. *Biomed. Biochim. Acta* **48**, 255–259 (1989).
9. Dewhirst, M. W., Braun, R. D. & Lanzen, J. L. Temporal changes in PO2 of R3230AC tumors in Fischer-344 rats. *Int J Radiat Oncol Biol Phys* **42**, 723–726 (1998)
10. Padera, T. P. *et al.* Pathology: cancer cells compress intratumour vessels. *Nature* **427**, 695 (2004).
11. Brown, J. M. & Wilson, W. R. Exploiting tumour hypoxia in cancer treatment. *Nature Rev. Cancer* **4**, 437–447 (2004).
12. Leu, A. J., Berk, D. A., Lymboussaki, A., Alitalo, K. & Jain, R. K. Absence of functional lymphatics within a murine sarcoma: a molecular and functional evaluation. *Cancer Res.* **60**, 4324–4327 (2000).
13. Jain, R. K., Munn, L. L. & Fukumura, D. Dissecting tumour pathophysiology using intravital microscopy. *Nature Rev. Cancer* **2**, 266–276 (2002).
14. Jain, R. K. Delivery of molecular and cellular medicine to solid yumors. *Microcirculation* **4**, 3–21 (1997).
15. Milosevic, M. F. *et al.* Interstitial fluid pressure in cervical carcinoma: within tumor heterogeneity, and relation to oxygen tension. *Cancer* **82**, 2418–2426 (1998).
16. Heldin, C. H., Rubin, K., Pietras, K. & Ostman, A. High interstitial fluid pressure — an obstacle in cancer therapy. *Nature Rev. Cancer* **4**, 806–813 (2004).
An excellent review of IFP in cancer.
17. Netti, P. A., Berk, D. A., Swartz, M. A., Grodzinsky, A. J. & Jain, R. K. Role of extracellular matrix assembly in interstitial transport in solid tumors. *Cancer Res.* **60**, 2497–2503 (2000).
18. Davies Cde, L., Berk, D. A., Pluen, A. & Jain, R. K. Comparison of IgG diffusion and extracellular matrix composition in rhabdomyosarcomas grown in mice versus *in vitro* as spheroids reveals the role of host stromal cells. *Br. J. Cancer* **86**, 1639–1644 (2002).
19. Brown, E. *et al.* Dynamic imaging of collagen and its modulation in tumors *in vivo* using second-harmonic generation. *Nature Med.* **9**, 796–800 (2003).
20. Jain, R. K. Barriers to drug delivery in solid tumours. *Sci. Amer.* **271**, 59–65 (1994).
21. Tannock, I. F. & Rotin, D. Acid pH in tumors and its potential for therapeutic exploitation. *Cancer Res.* **49**, 4373–4384 (1989).
22. Raghunand, N., Gatenby, R. A. & Gillies, R. J. Microenvironmental and cellular consequences of altered blood flow in tumours. *Br. J. Radiol.* **76 Spec No. 1**, S11–S22 (2003).
23. Gatenby, R. A. & Gillies, R. J. Why do cancers have high aerobic glycolysis? *Nature Rev. Cancer* **4**, 891–899 (2004).
24. Moulder, J. E. & Rockwell, S. Tumor hypoxia: its impact on cancer therapy. *Cancer Metastasis Rev.* **5**, 313–341 (1987).
25. Harris, A. L. Hypoxia — a key regulatory factor in tumour growth. *Nature Rev. Cancer* **2**, 38–47 (2002).
26. Dean, M., Fojo, T. & Bates, S. Tumour stem cells and drug resistance. *Nature Rev. Cancer* **5**, 275–284 (2005).
27. Tannock, I. F. The relation between cell proliferation and vascular system in a transplanted mouse mammary tumour. *Br. J. Cancer* **22**, 258–273 (1968).
A pivotal report on the gradient in cell proliferation kinetics as a function of distance from tumour vasculature.
28. Hirst, D. G. & Denekamp, J. Tumour cell proliferation in relation to the vasculature. *Cell Tissue Kinet.* **12**, 31–42 (1979).
29. Tannock, I. Cell kinetics and chemotherapy: a critical review. *Cancer Treat. Rep.* **62**, 1117–1133 (1978).
30. Brown, J. M. NCI's anticancer drug screening program may not be selecting for clinically active compounds. *Oncol. Res.* **9**, 213–215 (1997).
31. van der Greef, J. & McBurney, R. N. Innovation: Rescuing drug discovery: *in vivo* systems pathology and systems pharmacology. *Nature Rev. Drug Discov.* **4**, 961–967 (2005).
32. Allen, L., Kimura, K., MacKitchan, J. & Ritschel, W. A. Manual of symbols, equations and definitions in pharmacokinetics. *J. Clin. Pharmacol.* **22**, 1S-23S (1982).
33. Rentsch, K. M. *et al.* Pharmacokinetic studies of mitoxantrone and one of its metabolites in serum and urine in patients with advanced breast cancer. *Eur. J. Clin. Pharmacol.* **54**, 83–89 (1998).
34. Hashizume, H. *et al.* Openings between defective endothelial cells explain tumor vessel leakiness. *Am. J. Pathol.* **156**, 1363–1380 (2000).
35. Hobbs, S. K. *et al.* Regulation of transport pathways in tumor vessels: role of tumor type and microenvironment. *Proc. Natl Acad. Sci. USA* **95**, 4607–4612 (1998).
36. Yuan, F. *et al.* Vascular permeability in a human tumor xenograft: molecular size dependence and cutoff size. *Cancer Res.* **55**, 3752–3756 (1995).
37. Jain, R. K. Transport of molecules in the tumor interstitium: a review. *Cancer Res.* **47**, 3039–3051 (1987).
38. Crank, J. *The Mathematics of Diffusion* (Clarendon Press, Oxford, 1975).
39. Coley, H. M., Amos, W. B., Twentyman, P. R. & Workman, P. Examination by laser scanning confocal fluorescence imaging microscopy of subcellular localisation of anthracyclines in parent and multidrug resistant cell lines. *Br. J. Cancer* **67**, 1316–1323 (1993).
40. Lee, C. M. & Tannock, I. F. Inhibition of endosomal sequestration of basic anticancer drugs: influence on cytotoxicity and tissue penetration. *Br. J. Cancer* (2006).
41. Sutherland, R. M. & Durand, R. E. Radiation response of multicell spheroids — an *in vitro* tumour model. *Curr. Top. Radiat. Res. Q.* **11**, 87–139 (1976).
42. Sutherland, R. M. Cell and environment interactions in tumor microregions: the multicell spheroid model. *Science* **240**, 177–184 (1988).

43. Durand, R. E. Variable radiobiological responses of spheroids. *Radiat. Res.* **81**, 85–99 (1980).
44. Sutherland, R. M., Eddy, H. A., Bareham, B., Reich, K. & Vanantwerp, D. Resistance to adriamycin in multicellular spheroids. *Int. J. Radiat. Oncol. Biol. Phys.* **5**, 1225–1230 (1979).
45. Kerr, D. J. & Kaye, S. B. Aspects of cytotoxic drug penetration, with particular reference to anthracyclines. *Cancer Chemother. Pharmacol.* **19**, 1–5 (1987).
46. Kerr, D. J., Wheldon, T. E., Hydns, S. & Kaye, S. B. Cytotoxic drug penetration studies in multicellular tumour spheroids. *Xenobiotica* **18**, 641–648 (1988).
47. Durand, R. E. Slow penetration of anthracyclines into spheroids and tumors: a therapeutic advantage? *Cancer Chemother. Pharmacol.* **26**, 198–204 (1990).
48. West, G. W., Weichselbaum, R. & Little, J. B. Limited penetration of methotrexate into human osteosarcoma spheroids as a proposed model for solid tumor resistance to adjuvant chemotherapy. *Cancer Res.* **40**, 3665–3668 (1980).
49. Wibe, E. Resistance to vincristine of human cells grown as multicellular spheroids. *Br. J. Cancer* **42**, 937–941 (1980).
50. Nederman, T., Carlsson, J. & Malmqvist, M. Penetration of substances into tumor tissue — a methodological study on cellular spheroids. *In Vitro* **17**, 290–298 (1981).
51. Nederman, T. & Carlsson, J. Penetration and binding of vinblastine and 5-fluorouracil in cellular spheroids. *Cancer Chemother. Pharmacol.* **13**, 131–135 (1984).
52. Sutherland, R., Buchegger, F., Schreyer, M., Vacca, A. & Mach, J. P. Penetration and binding of radiolabeled anti-carcinoembryonic antigen monoclonal antibodies and their antigen binding fragments in human colon multicellular tumor spheroids. *Cancer Res.* **47**, 1627–1633 (1987).
53. Mairs, R. J., Angerson, W. J., Babich, J. W. & Murray, T. Differential penetration of targeting agents into multicellular spheroids derived from human neuroblastoma. *Prog. Clin. Biol. Res.* **366**, 495–501 (1991).
54. Erlanson, M., Daniel-Szolgay, E. & Carlsson, J. Relations between the penetration, binding and average concentration of cytostatic drugs in human tumour spheroids. *Cancer Chemother. Pharmacol.* **29**, 343–353 (1992).
55. Erlichman, C. & Vidgen, D. Cytotoxicity of adriamycin in MGH-U1 cells grown as monolayer cultures, spheroids, and xenografts in immune-deprived mice. *Cancer Res.* **44**, 5369–5375 (1984).
56. Durand, R. E. Use of Hoechst 33342 for cell selection from multicell systems. *J. Histochem. Cytochem.* **30**, 117–122 (1982).
Description of an important flow cytometry method whereby tumour cells can be sorted from tumours on the basis of their proximity to blood vessels.
57. Durand, R. E. Chemosensitivity testing in V79 spheroids: drug delivery and cellular microenvironment. *J. Natl Cancer Inst.* **77**, 247–252 (1986).
58. Olive, P. L. & Durand, R. E. Drug and radiation resistance in spheroids: cell contact and kinetics. *Cancer Metastasis Rev.* **13**, 121–138 (1994).
59. Oloumi, A., Lam, W., Banath, J. P. & Olive, P. L. Identification of genes differentially expressed in V79 cells grown as multicell spheroids. *Int. J. Radiat. Biol.* **78**, 483–492 (2002).
60. Teicher, B. A. *et al.* Tumor resistance to alkylating agents conferred by mechanisms operative only *in vivo*. *Science* **247**, 1457–1461 (1990).
61. Kobayashi, H. *et al.* Acquired multicellular-mediated resistance to alkylating agents in cancer. *Proc. Natl Acad. Sci. USA* **90**, 3294–3298 (1993).
62. Hicks, K. O. *et al.* An experimental and mathematical model for the extravascular transport of a DNA intercalator in tumours. *Br. J. Cancer* **76**, 894–903 (1997).
63. Minchinton, A. I., Wendt, K. R., Clow, K. A. & Fryer, K. H. Multilayers of cells growing on a permeable support: an *in vitro* tumour model. *Acta Oncologica* **36**, 13–16 (1997).
64. Cowan, D. S. M., Hicks, K. O. & Wilson, W. R. Multicellular membranes as an *in vitro* model for extravascular diffusion in tumours. *Br. J. Cancer* **74**, S28–S31 (1996).
65. Hicks, K. O., Fleming, Y., Siim, B. G., Koch, C. J. & Wilson, W. R. Extravascular diffusion of tirapazamine: Effect of metabolic consumption assessed using the multicellular layer model. *Int. J. Radiat. Oncol. Biol. Phys.* **42**, 641–649 (1998).
66. Tannock, I. F., Lee, C. M., Tunggal, J. K., Cowan, D. S. & Egorin, M. J. Limited penetration of anticancer drugs through tumor tissue: a potential cause of resistance of solid tumors to chemotherapy. *Clin. Cancer Res.* **8**, 878–884. (2002).
67. Phillips, R. M., Loadman, P. M. & Cronin, B. P. Evaluation of a novel *in vitro* assay for assessing drug penetration into avascular regions of tumours. *Br. J. Cancer* **77**, 2112–2119 (1998).
68. Tunggal, J. K., Cowan, D. S., Shaikh, H. & Tannock, I. F. Penetration of anticancer drugs through solid tissue: a factor that limits the effectiveness of chemotherapy for solid tumors. *Clin. Cancer Res.* **5**, 1583–1586 (1999).
69. Kyle, A. H., Huxham, L. A., Chiam, A. S., Sim, D. H. & Minchinton, A. I. Direct assessment of drug penetration into tissue using a novel application of three-dimensional cell culture. *Cancer Res.* **64**, 6304–6309 (2004).
70. Hicks, K. O., Pruijn, F. B., Baguley, B. C. & Wilson, W. R. Extravascular transport of the DNA intercalator and topoisomerase poison N-[2-(Dimeth ylamino)ethyl]acridine-4-carboxamide (DACA): diffusion and metabolism in multicellular layers of tumor cells. *J. Pharmacol. Exp. Ther.* **297**, 1088–1098 (2001).
71. Hicks, K. O., Pruijn, F. B., Sturman, J. R., Denny, W. A. & Wilson, W. R. Multicellular resistance to tirapazamine is due to restricted extravascular transport: a pharmacokinetic/pharmacodynamic study in HT29 multicellular layer cultures. *Cancer Res.* **63**, 5970–5977 (2003).
72. Kyle, A. & Minchinton, A. I. Measurement of delivery and metabolism of tirapazamine through tumour tissue using the multilayered cell culture model. *Cancer Chemother. Pharmacol.* **43**, 213–220 (1999).
73. Wilson, W. R. & Hicks, K. O. Measurement of extravascular drug diffusion in multicellular layers. *Br. J. Cancer* **79**, 1623–1626 (1999).
74. Grantab, R., Sivananthan, S. & Tannock, I. F. The penetration of anticancer drugs through tumor tissue as a function of cellular adhesion and packing density of tumor cells. *Cancer Res.* **66**, 1033–1039 (2006).
75. Goldacre, R. J. & Sylvén, B. A rapid method for studying tumour blood supply using systemic dyes. *Nature* **184**, 63–64 (1959).
76. Goldacre, R. J. & Sylvén, B. On the access of blood-borne dyes to various tumour regions. *Br. J. Cancer* 16, 306–322 (1962).
Excellent and comprehensive review of early studies (pre 1960) of tumour vasculature and their relevance to drug delivery.
77. Egorin, M. J., Hildebrand, R. C., Cimino, E. F. & Bachur, N. R. Cytofluorescence localisation of adriamycin and daunomycin. *Cancer Res.* **34**, 2243–2245 (1974).
78. Ozols, R. F. *et al.* Pharmacokinetics of adriamycin and tissue penetration in murine ovarian cancer. *Cancer Res.* **39**, 3209–3214 (1979).
79. Henneberry, H. P. & Aherne, G. W. Visualisation of doxorubicin in human and animal tissues and in cell cultures by immunogold-silver staining. *Br. J. Cancer* **65**, 82–86 (1992).
80. Henneberry, H. P., Aherene, G. W. & Marks, V. Immunocytochemical localisation of VP16–213 in normal and malignant tissues. *Cancer Lett.* **37**, 225–233 (1987).
81. Jain, R. K., Weissbrod, J. M. & Wei, J. Mass transport in tumors: characterization and applications to chemotherapy. *Adv. Cancer Res.* **33**, 251–310 (1980).
82. Simpson-Herren, L., Noker, P. E. & Wagoner, S. D. Variability of tumor response to chemotherapy. II. Contribution of tumor heterogeneity. *Cancer Chemother. Pharmacol.* **22**, 131–136 (1988).
83. Simpson-Herren, L. & Noker, P. E. Diversity of penetration of anti-cancer agents into solid tumours. *Cell Prolif.* **24**, 355–365 (1991).
84. Chaplin, D. J., Durand, R. E. & Olive, P. L. Cell selection from a murine tumour using the fluorescent probe Hoechst 33342. *Br. J. Cancer* **51**, 569–572 (1985).
85. Gerlowski, L. E. & Jain, R. K. Microvascular permeability of normal and neoplastic tissues. *Microvasc. Res.* **31**, 288–305 (1986).
86. Dewhirst, M. W. *et al.* Perivascular oxygen tensions in a transplantable mammary tumor growing in a dorsal flap window chamber. *Radiat. Res.* **130**, 171–182 (1992).
87. Leunig, M. *et al.* Angiogenesis, microvascular architecture, microhemodynamics, and interstitial fluid pressure during early growth of human adenocarcinoma LS174T in SCID mice. *Cancer Res.* **52**, 6553–6560 (1992).
88. Wu, N. Z., Klitzman, B., Rosner, G., Needham, D. & Dewhirst, M. W. Measurement of material extravasation in microvascular networks using fluorescence video-microscopy. *Microvasc. Res.* **46**, 231–253 (1993).
89. Helmlinger, G., Yuan, F., Dellian, M. & Jain, R. K. Interstitial pH and pO2 gradients in solid tumors *in vivo*: high-resolution measurements reveal a lack of correlation. *Nature Med.* **3**, 177–182 (1997).
90. Dreher, M. R. *et al.* Tumor vascular permeability, accumulation, and penetration of macromolecular drug carriers. *J. Natl Cancer Inst.* **98**, 335–344 (2006).
91. Brown, E. B. *et al.* *In vivo* measurement of gene expression, angiogenesis and physiological function in tumors using multiphoton laser scanning microscopy. *Nature Med.* **7**, 864–868 (2001).
92. Tong, R. T. *et al.* Vascular normalization by vascular endothelial growth factor receptor 2 blockade induces a pressure gradient across the vasculature and improves drug penetration in tumors. *Cancer Res.* **64**, 3731–3736 (2004).
93. Winkler, F. *et al.* Kinetics of vascular normalization by VEGFR2 blockade governs brain tumor response to radiation: role of oxygenation, angiopoietin-1, and matrix metalloproteinases. *Cancer Cell* **6**, 553–563 (2004).
94. Willett, C. G. *et al.* Direct evidence that the VEGF-specific antibody bevacizumab has antivascular effects in human rectal cancer. *Nature Med.* **10**, 145–147 (2004).
95. Kuh, H. J., Jang, S. H., Wientjes, M. G., Weaver, J. R. & Au, J. L. Determinants of paclitaxel penetration and accumulation in human solid tumor. *J. Pharmacol. Exp. Ther.* **290**, 871–880 (1999).
96. Jang, S. H., Wientjes, M. G., Lu, D. & Au, J. L. Drug delivery and transport to solid tumors. *Pharm. Res.* **20**, 1337–1350 (2003).
97. Terheggen, P. M. *et al.* Immunocytochemical detection of interaction products of cis-diamminedichloroplatinum(II) and cis-diammine(1,1-cyclobutanedicarboxylato) platinum(II) with DNA in rodent tissue sections. *Cancer Res.* **47**, 6719–6725 (1987).
98. Meijer, C. *et al.* Immunocytochemical analysis of cisplatin-induced platinum-DNA adducts with double-fluorescence video microscopy. *Br. J. Cancer* **76**, 290–298 (1997).
99. Raleigh, J. A. *et al.* Hypoxia and vascular endothelial growth factor expression in human squamous cell carcinomas using pimonidazole as a hypoxia marker. *Cancer Res.* **58**, 3765–3768 (1998).
100. Huxham, L. A., Kyle, A. H., Baker, J. H. E., McNicol, K. L. & Minchinton, A. I. Tirapazamine causes vascular dysfunction in HCT-116 tumour xenografts. *Radiother. Oncol.* **78**, 138–145 (2006).
101. Lankelma, J. *et al.* Doxorubicin gradients in human breast cancer. *Clin. Cancer Res.* **5**, 1703–1707 (1999).
102. Primeau, A. J., Rendon, A., Hedley, D., Lilge, L. & Tannock, I. F. The distribution of the anticancer drug Doxorubicin in relation to blood vessels in solid tumors. *Clin. Cancer Res.* **11**, 8782–8788 (2005).
103. Koch, C. J., Evans, S. M. & Lord, E. M. Oxygen dependence of cellular uptake of EF5 [2-(2-nitro-1H-imidazol-1-yl)-N-(2, 2, 3, 3, 3-pentafluoropropyl)a cet amide]: analysis of drug adducts by fluorescent antibodies vs bound radioactivity. *Br. J. Cancer* **72**, 869–874 (1995).
104. Huxham, L. A., Kyle, A. H., Baker, J. H., Nykilchuk, L. K. & Minchinton, A. I. Microregional effects of gemcitabine in HCT-116 xenografts. *Cancer Res.* **64**, 6537–6541 (2004).
105. Wu, L. & Tannock, I. F. Effect of the selective estrogen receptor modulator arzoxifene on repopulation of hormone-responsive breast cancer xenografts between courses of chemotherapy. *Clin. Cancer Res.* **11**, 8195–8200 (2005).
106. Kyle, A. H., Huxham, L. A., Baker, J. H., Burston, H. E. & Minchinton, A. I. Tumor distribution of bromodeoxyuridine-labeled cells is strongly dose dependent. *Cancer Res.* **63**, 5707–5711 (2003).
107. Sherar, M. D. Imaging in Oncology. in *The Basic Science of oncology* (eds Tannock, I. F., Hill, R. P., Bristow, R. G. & Harrington, L.) 249–260 (McGrow-Hill, New York, USA, 2005).
108. Gambhir, S. S. Molecular imaging of cancer with positron emission tomography. *Nature Rev. Cancer* **2**, 683–693 (2002).

109. Durand, R. E. Flow cytometry studies of intracellular adriamycin in multicell spheroids *in vitro*. *Cancer Res.* **41**, 3495–3498 (1981).
110. Kim, J. J. & Tannock, I. F. Repopulation of cancer cells during therapy: an important cause of treatment failure. *Nature Rev. Cancer* **5**, 516–525 (2005).
111. Brown, J. M. SR 4233 (Tirapazamine): a new anticancer drug exploiting hypoxia in solid tumours. *Br. J. Cancer* **67**, 1163–1170 (1993).
112. Rischin, D. *et al.* Phase I trial of concurrent tirapazamine, cisplatin, and radiotherapy in patients with advanced head and neck cancer. *J. Clin. Oncol.* **19**, 535–542 (2001).
113. Rischin, D. *et al.* Tirapazamine, Cisplatin, and Radiation versus Fluorouracil, Cisplatin, and Radiation in patients with locally advanced head and neck cancer: a randomized phase II trial of the Trans-Tasman Radiation Oncology Group (TROG 98. 02). *J. Clin. Oncol.* **23**, 79–87 (2005).
114. Hicks, K. O., Siim, B. G., Pruijn, F. B. & Wilson, W. R. Oxygen dependence of the metabolic activation and cytotoxicity of tirapazamine: implications for extravascular transport and activity in tumors. *Radiat. Res.* **161**, 656–666 (2004).
115. Baxter, L. T., Zhu, H., Mackensen, D. G., Butler, W. F. & Jain, R. K. Biodistribution of monoclonal antibodies: scale-up from mouse to human using a physiologically based pharmacokinetic model. *Cancer Res.* **55**, 4611–4622 (1995).
116. Piccart-Gebhart, M. J. *et al.* Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. *N. Engl. J. Med.* **353**, 1659–1672 (2005).
117. Romond, E. H. *et al.* Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. *N. Engl. J. Med.* **353**, 1673–1684 (2005).
118. Vogel, C. L. *et al.* Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer. *J. Clin. Oncol.* **20**, 719–726 (2002).
119. Izumi, Y., Xu, L., di Tomaso, E., Fukumura, D. & Jain, R. K. Tumour biology: herceptin acts as an anti-angiogenic cocktail. *Nature* **416**, 279–280. (2002).
120. Pruijn, F. B. *et al.* Extravascular transport of drugs in tumor tissue: effect of lipophilicity on diffusion of tirapazamine analogues in multicellular layer cultures. *J. Med. Chem.* **48**, 1079–1087 (2005).
121. Siim, B. G. *et al.* Hypoxia-selective 3-alkyl-1, 2, 4-benzotriazine 1, 4-dioxide bioreductive drugs: Analogues of tirapazamine with improved *in vivo* activity. *Clin. Cancer Res.* **11**, 8973s (2005).
122. Hicks, K. O. *et al.* Penetration of three dimensional tissue culture predicts *in vivo* activity of hypoxia-targeted anticancer drugs. *J. Natl Cancer. Inst.* (in the press)
123. Minchinton, A. I., Durand, R. E. & Chaplin, D. J. Intermittent blood flow in the KHT sarcoma- flow cytometry studies using Hoechst 33342. *Br. J. Cancer* **62**, 195–200 (1990).
124. Kotelnikov, V. M. *et al. In vivo* labelling with halogenated pyrimidines of squamous cell carcinomas and adjacent non-involved mucosa of head and neck region. *Cell Prolif.* **28**, 497–509 (1995).
125. Boucher, Y. & Jain, R. K. Microvascular pressure is the principle driving force for interstitial hypertension in solid tumours: Implications for vascular collapse. *Cancer Res.* **52**, 5110–5114 (1992).
126. Jain, R. K. Molecular regulation of vessel maturation. *Nature Med.* **9**, 685–693 (2003).
A review of vascular structure and architecture within tumours, and a discussion of vessel normalization phenomenon.
127. Hurwitz, H. *et al.* Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *N. Engl. J. Med.* **350**, 2335–2342 (2004).
128. Jain, R. K. Normalizing tumor vasculature with anti-angiogenic therapy: a new paradigm for combination therapy. *Nature Med.* **7**, 987–989 (2001).
129. Franco, M. *et al.* Targeted anti-vascular endothelial growth factor receptor-2 therapy leads to short-term and long-term impairment of vascular function and increase in tumor hypoxia. *Cancer Res.* **66**, 3639–3648 (2006).
130. Riesterer, O. *et al.* Ionizing radiation antagonizes tumor hypoxia induced by antiangiogenic treatment. *Clin. Cancer Res.* **12**, 3518–3524 (2006).
131. Cowan, D. S. & Tannock, I. F. Factors that influence the penetration of methotrexate through solid tissue. *Int. J. Cancer* **91**, 120–125 (2001).
132. Tunggal, J. K., Melo, T., Ballinger, J. R. & Tannock, I. F. The influence of expression of P-glycoprotein on the penetration of anticancer drugs through multicellular layers. *Int. J. Cancer* **86**, 101–107 (2000).
133. Milroy, R. A randomised clinical study of verapamil in addition to combination chemotherapy in small cell lung cancer. West of Scotland Lung Cancer Research Group, and the Aberdeen Oncology Group. *Br. J. Cancer* **68**, 813–818 (1993).
134. Wishart, G. C. *et al.* Quinidine as a resistance modulator of epirubicin in advanced breast cancer: mature results of a placebo-controlled randomized trial. *J. Clin. Oncol.* **12**, 1771–1777 (1994).
135. Kerbel, R. S., St Croix, B., Florenes, V. A. & Rak, J. Induction and reversal of cell adhesion-dependent multicellular drug resistance in solid breast tumors. *Hum. Cell* **9**, 257–264 (1996).
136. St Croix, B., Man, S. & Kerbel, R. S. Reversal of intrinsic and acquired forms of drug resistance by hyaluronidase treatment of solid tumors. *Cancer Lett.* **131**, 35–44 (1998).
137. Jang, S. H., Wientjes, M. G. & Au, J. L. Enhancement of paclitaxel delivery to solid tumors by apoptosis-inducing pretreatment: effect of treatment schedule. *J. Pharmacol. Exp. Ther.* **296**, 1035–1042 (2001).
138. Griffon-Etienne, G., Boucher, Y., Brekken, C., Suit, H. D. & Jain, R. K. Taxane-induced apoptosis decompresses blood vessels and lowers interstitial fluid pressure in solid tumors: clinical implications. *Cancer Res.* **59**, 3776–3782 (1999).
139. McKee, T. D. *et al.* Degradation of fibrillar collagen in a human melanoma xenograft improves the efficacy of an oncolytic herpes simplex virus vector. *Cancer Res.* **66**, 2509–2513 (2006).
140. Mayer, L. D., Bally, M. B. & Cullis, P. R. Uptake of adriamycin into large unilamellar vesicles in response to a pH gradient. *Biochim. Biophys. Acta* **857**, 123–126 (1986).
141. Millot, C., Millot, J. M., Morjani, H., Desplaces, A. & Manfait, M. Characterization of acidic vesicles in multidrug-resistant and sensitive cancer cells by acridine orange staining and confocal microspectrofluorometry. *J. Histochem. Cytochem.* **45**, 1255–1264 (1997).
142. Poole, B. & Ohkuma, S. Effect of weak bases on the intralysosomal pH in mouse peritoneal macrophages. *J. Cell. Biol.* **90**, 665–669 (1981).
143. Mattsson, J. P., Vaananen, K., Wallmark, B. & Lorentzon, P. Omeprazole and bafilomycin, two proton pump inhibitors: differentiation of their effects on gastric, kidney and bone H(+)-translocating ATPases. *Biochim. Biophys. Acta* **1065**, 261–268 (1991).
144. Vail, D. M. *et al.* Pegylated liposomal doxorubicin: proof of principle using preclinical animal models and pharmacokinetic studies. *Semin. Oncol.* **31**, 16–35 (2004).
145. Nyman, D. W. *et al.* Phase I and pharmacokinetics trial of ABI-007, a novel nanoparticle formulation of paclitaxel in patients with advanced nonhematologic malignancies. *J. Clin. Oncol.* **23**, 7785–7793 (2005).

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Competing interests statement
The authors declare competing financial interests: see web version for details.

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