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ORIGINAL ARTICLE

Accumulation and distribution of doxorubicin in tumour spheroids: the influence of acidity and expression of P-glycoprotein

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Abstract
Purpose The intra-tumour distribution of anticancer drugs remains an important, but often under-estimated, influence on drug efficacy. Tumour acidity and the presence of efflux pumps were examined for their influence on the distribution of doxorubicin in a solid tumour model.
Methods Anticancer drug distribution and overall accumulation was measured in tumour spheroids (TS) of varying sizes. The distribution profiles were examined in normoxic and hypoxic TS, the latter generating metabolic acidosis. Finally, the drug distribution profiles were related to efficacy using radial outgrowth assays.
Results In large tumour spheroids (TS) (d $\sim$500 μm), intracellular accumulation of doxorubicin was restricted to cells in the outermost layers and failed to accumulate within the viable cells in the 'intermediate' hypoxic zone. A similar profile was obtained for another protonatable amine, 7-AAD. In contrast, the distribution of the non-ionisable drug (at physiological pH) BODIPY-Taxol was uniform throughout the TS. In order to independently model the hypoxic and normoxic zones of TS, we compared drug accumulation in small entirely normoxic TS (d $\sim$200 μm) with equivalent sized ones exposed to hypoxia in an anaerobic chamber. Exposure of TS to hypoxia caused a considerable reduction in the pH of the bathing medium and lower tissue accumulation of doxorubicin. Interstitial acidity reduces the proportion of doxorubicin in the non-ionised form.
Conclusions In TS, the accumulation and distribution of doxorubicin was influenced by both the expression of P-glycoprotein and hypoxia-induced acidity. Therefore, optimisation of doxorubicin chemotherapy for hypoxic tumours will require circumvention of both of these crucial pharmacokinetic determinants.

Keywords P-glycoprotein · Doxorubicin · Solid tumours · Bioenergetic metabolism · Tumour spheroid · Cancer chemotherapy · Drug resistance · Hypoxia · Tumour acidity

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Introduction

Chemotherapy of cancer with genotoxic drugs remains a mainstay of therapeutic intervention in front-line, adjuvant and palliative situations. Unfortunately, the efficacy of cancer chemotherapy is poor towards many solid tumours from the outset, or following initial success, the tissue presents a seemingly insurmountable barrier [1–3]. This resistance of tumours to chemotherapeutic drugs is a complex phenomenon with a number of factors responsible for negating genotoxic drug activity [3, 4]. Clearly, the inherent sensitivity of cancer cells to a particular anticancer drug is a key factor in determining overall efficacy. However, achieving sufficient levels of drug throughout the tumour and within target cells is the major initial barrier.

Cellular susceptibility to anticancer drugs will be influenced by a number of resistance modifiers that may be inherently expressed or appear only following exposure to chemotherapy. For example, adoption of a quiescent state will severely reduce the impact of conventional genotoxic drugs, which target different aspects of cell proliferation [5–7]. Upregulation of DNA repair systems will limit the

effectiveness of many anticancer drugs including cisplatin and cyclophosphamide [8–10]. Aberrant cellular signalling pathways will alter the balance between undergoing apoptosis and promoting repair of damage [11–13]. Similarly, mutations in protein-based drug targets (e.g. topoisomerase, tubulin) may prevent anticancer drugs eliciting their damaging effects [14–17].

The above cellular alterations are products of random and stress-induced mutations in cancer cells and thereby represent an adaptive form of resistance to chemotherapy. The 3-D arrangement of cells and vessels within a tumour significantly impacts on local drug pharmacokinetic profiles, often resulting in an intrinsic resistance phenotype [18, 19]. Studies using clinical tissue and in vivo animal models have demonstrated that the ability of drugs to penetrate avascular regions of often >150 μm varies with their chemico-physical characteristics and the nature of the interstitial channels within the tissue [20–22]. In particular, hydrostatic pressure driving drug convection along a sizeable concentration gradient towards the central tumour regions is offset by high cell density and significant interstitial pressure [22, 23]. The balance of these forces greatly impairs the ability or time-course for drug penetration through the avascular regions of solid tumours. The degree of cellular uptake will also influence distribution through these regions. For example, animal model studies have demonstrated that avid uptake in cells proximal to the blood vessels would lower the extracellular drug concentration, thereby reducing the convective force; conversely, poor uptake into these regions would promote more extensive distribution [24, 25].

Uptake into cells is a key factor in chemotherapy of solid tumours since it will obviously influence the ability to reach the intracellular target and, as mentioned earlier, contribute to the overall tissue distribution. Uptake into cells is dependent on (1) lipophilicity of drugs, (2) necessity of an uptake pump [26–28], (3) the presence of efflux pumps [24, 29, 30], and (4) micro-environmental factors that alter drug ionisation status (for review see [4]). Perhaps, the most established and widely investigated resistance pathway involves the triad of drug efflux pumps from the ATP-binding cassette (ABC) family [24, 29, 30]. Two of the pumps (P-gp or ABCB1, and ABCG2) appear to extract drugs directly from the bilayer and mediate their expulsion in an ATP-dependent manner. The third member of the triad (i.e. MRP1 or ABCC1) primarily mediates the removal of secondary drug metabolites, namely the sulphate and glutathione conjugates [31–33]. These pumps display distinct, but overlapping, specificities and have been associated with distinct cancer types.

The vast majority of anticancer drugs are lipophilic, thereby enabling rapid diffusion across the plasma membrane. Only the non-ionised form of such drugs may permeate the membrane; however, a number of drugs at physiological pH are present in an ionised form [34]. The latter arises due to pKa values for the compounds near the physiological range. This is of particular importance in the central, or avascular, regions of solid tumours where the interstitial pH is usually acidic and the deviation from physiological values will impact on the ratio of ionised/non-ionised drug. Acidification of the interstitial pH results from the build-up of carbonic acid from CO_2 due to aerobic metabolism and lactic acid through anaerobic metabolism [35, 36]. The Na/H-exchanger (NHE), the monocarboxylate transporter (MCT) and carbonic anhydrase IX (CA IX) [38–40] proteins ensure extrusion of these acidic molecules to the interstitium.

The influences of efflux pumps and interstitial acidification on the distribution and cellular uptake of certain chemotherapeutic drugs in solid tumours are not independent. Hypoxia has emerged as a causal link between these two key factors. Hypoxia leads to stabilisation of the transcription factor 1-alpha (Hif1α), which alters bioenergetic metabolism and produces acidification. It is also well established that Hif1α also induces expression of P-gp, which is an essential element of the cellular stress response [41, 42]. Therefore, the emergence of hypoxia in the avascular regions of a solid tumour will impact on drug pharmacokinetics via both of these mechanisms. The anthracycline anticancer drugs (e.g. doxorubicin) have pKa values in the near physiological range [34] and are substrates for transport by P-gp [24, 43]. The purpose of this investigation is to ascertain whether doxorubicin distribution, and cellular uptake, is compromised in large tumour spheroids (TS) that contain regions of hypoxia. TS provide useful models of avascular tumour regions, containing both normoxic and hypoxic zones when grown beyond 200 μm in diameter. Here, various TS models have been utilised and developed to enable systematic assessment of the importance of hypoxia and P-gp expression on the distribution of doxorubicin. The TS is an ideal model to represent the avascular regions of solid tumours and mimics many of the micro-environmental factors.

Materials and methods

Materials

RPMI 1640 medium (with GlutaMAX I w/o HEPES), Fetal Bovine Serum (Heat Inactivated), Penicillin/Streptomycin solution and trypsin–EDTA were purchased from Life Technologies Ltd. Doxorubicin hydrochloride was obtained from Sigma, UK. The anaerobic chamber and Anaero*Gen*™ sachets were obtained from Oxoid, UK.

Cell lines, culture and TS production

DLD-1 (colorectal adenocarcinoma), HT29 (colon adenocarcinoma) and NCI^{ADR} (ovarian adenocarcinoma) cell lines were obtained from Dr. Roger Phillips, University of Bradford, UK. All cell lines were cultured in RPMI 1640 medium supplemented with 10% (v/v) foetal bovine serum and penicillin (100 IU/ml)/streptomycin (100 mg/ml). Large TS (~500 μm) were grown in spinner flasks as described in [44]. Small TS were prepared using the liquid overlay technique in 96-well tissue culture plates coated with 100 μl of 0.75% (w/v) agarose prepared in RPMI-1640 medium (w/o FBS). Freshly trypsinised cells taken from exponentially growing cultures were overlaid on the agarose base coats at a density of 20×10^3 cells in 100 μl RPMI 1640 medium. The cells were kept stationary for 24 h (DLD-1) or 48 h (HT29, NCI^{ADR}) at 37°C/5% CO_2 to allow the TS to form. The plates were then transferred to a Titramax 100 (Heidolph Instruments) and shaken at 300 rpm at 37°C/5% CO_2 for a further 24 h.

Hypoxic incubations

Hypoxic incubations (0.1% O_2) were carried out at 37°C in a 3.5-l anaerobic chamber containing an Anaero*Gen*™ sachet. O_2 levels within the jar were monitored using a calibrated Mini O2DII oxygen sensor (Analox, UK) [45].

Doxorubicin accumulation in TS

TS were pre-exposed to hypoxia/normoxia (5% CO_2) at 37°C for 24 h prior to drug administration. Doxorubicin was prepared in medium at twice the desired concentration to give a final concentration range of 100 nM–100 μM when 100-μl aliquots were added to each well. For P-gp inhibition studies, Tariquidar (500 nM) was also included in the culture medium. The TS were then exposed to hypoxia/normoxia (5% CO_2) for a further 16 h in the presence of the drug(s). At the end of the incubation period, TS ($n = 6$) were removed to 0.5-ml tubes, the medium was aspirated, and the TS were washed with 200 μl of PBS. The TS were then solubilised in 200 μl of 2% SDS for 72 h at 25°C. An aliquot of the sample (100 μl) was transferred to a 96-well plate, and the fluorescence ($\lambda_{ex} = 485 \pm 5$ nm, $\lambda_{em} = 538 \pm 5$ nm) was measured using a SpectraMax Gemini XPS spectrofluorometer (Molecular Devices, UK). The doxorubicin concentration of the samples was calculated from a standard curve of doxorubicin (100 nM–20 μM) in 2% SDS, which was always done at the same time. Another aliquot of the sample (20 μl) was taken for protein determination using the Bio-Rad DC protein assay (Bio-Rad, UK), and the amount of doxorubicin in the TS (nmol/mg protein) was calculated.

Fluorescence microscopy of TS—distribution of doxorubicin and BODIPY-Taxol

Confocal fluorescence microscopy was used to measure the distribution of doxorubicin and BODIPY-Taxol in TS using a Zeiss LSM510 confocal laser-scanning microscope (Carl Zeiss, Welwyn Garden City, UK). Doxorubicin and BODIPY-Taxol were excited using an argon laser ($\lambda_{ex} = 488$ nm) and detected using an emission filter set at 505–530 nm. TS were exposed to doxorubicin (100 μM) or BODIPY-Taxol (100 nM) for 16 h. Following drug incubation, sections were prepared from TS using a modified version of a previously used technique [25]. Briefly, TS were placed into OCT (RA Lamb Ltd., UK), snap-frozen in liquid nitrogen, and then stored at −80°C. Sections were cut (10 μm) by cryostat and mounted on glass slides prior to overnight air-drying, followed by confocal imaging.

Measurement of pH

The pH of the TS culture medium was measured using a HI 1083B micro-tipped pH electrode (Hanna Instruments, UK), which was calibrated using 'Colourkey' buffer solution pH 7.0 (VWR International, UK). All readings were taken immediately after normoxic/hypoxic incubations to avoid pH changes in the medium caused by equilibration to atmospheric CO_2. Measurements were taken in individual wells with the result of each experiment being the mean of six replicates. Control readings were performed on medium exposed to hypoxia/normoxia from wells without TS.

Doxorubicin cytotoxicity in TS

Hypoxic exposures and doxorubicin dosing of TS were performed as for the accumulation assay described earlier. After the 16-h incubation in the presence of doxorubicin (10 nM–100 μM), individual TS were moved to a new 'uncoated' well in a 48-well plate and 500 μl of fresh medium was added. The TS were incubated for 6 days to allow cellular outgrowth from the tissue. Following the incubation period, the medium was aspirated and replaced with 200 μl 5 g/L methylene blue in methanol to fix and stain the cells. The wells were washed five times with tap water, and the diameter of the outgrowth, including the original TS, was measured using a graduated microscope eyepiece graticule (Pyser-SGI, UK). The diameter was expressed as a percentage of that obtained in the absence of doxorubicin treatment. To obtain a quantitative measurement of the total number of cells present, the methylene blue–stained tissue was solubilised in 200 μl of 2% SDS overnight on a shaker at room temperature. A 100-μl aliquot was taken from each well and placed in the well of a

96-well plate, and the absorbance was measured at 650 nm using a Spectra Max 250 microplate reader.

Immunohistochemistry of TS—detection of hypoxic regions

Hypoxic regions in TS were identified by detection of pimonidazole adducts as previously described [45]. Briefly, prior to exposure to hypoxia, 100 mM of pimonidazole hydrochloride was added to the medium containing the TS. Following, hypoxic incubation, the tissues were fixed and sectioned. The primary antibody, hypoxyprobe-1, was used to detect pimonidazole adducts. A Mach 2 goat-anti-mouse HRP conjugate (BioCarta, Europe) was used as the secondary antibody and detection was achieved using DAB substrate chromogen (DakoCytomation, UK). Sections were counterstained with haematoxylin and mounted with aquamount.

Data analysis

Non-linear regression analyses were generated using the GraphPad Prism4.0 program. Statistical analyses between data sets were done using the Student's *t* test, and statistical significance was achieved where $P < 0.05$. Comparisons of mean ± SEM were undertaken with at least three independent observations in each case. The following hyperbolic equation was used to describe the accumulation of doxorubicin in TS in the presence of varying doses of drug:

$$B = \frac{B_{\max} \cdot [L]}{K_{\mathrm{d}} + [L]} \quad (1)$$

where B = amount of doxorubicin accumulated (nmol/mg protein); $B_{\max}$ = maximal accumulation; K_{d} = dissociation constant (nM); and $[L]$ = doxorubicin concentration added to medium.

The dose–response equation below was used to assess the effects of doxorubicin on radial outgrowth from TS:

$$B = B_{\min} + \frac{(B_{\max} - B_{\min})}{\left(1 + 10^{((\log IC_{50} - L)^{n})}\right)} \quad (2)$$

where B = radius of outgrowth; $B_{\max}$ = radius in the absence of doxorubicin; $B_{\min}$ = minimum radius, IC_{50} = concentration of drug that leads to half maximal reduction in radius; n = hill slope factor; and $L = \log_{10}$[doxorubicin].

Results

Drug accumulation in large TS

TS were grown from HT29 cells for a period of approximately 10 days at which time typical diameters were ~500 μm. TS of this size display a small central necrotic core formed due to the hostile internal micro-environment. As shown in Fig. 1a, the central region of TS is surrounded by a hypoxic region, which was identified by staining of pimonidazole adducts in tissue sections.

Accumulation and distribution within the HT29 TS was measured for two anticancer drugs: the anthracycline doxorubicin (Fig. 1b) and the paclitaxel derivative BODIPY-Taxol (Fig. 1d). Both compounds are fluorescent, thereby permitting detection using confocal microscopy. Confocal imaging of tissue sections taken from the centre of large spheroids (d = ~500 μm) avoids attenuation issues associated with imaging deep cell layers of intact spheroids of this size. Prolonged incubation (i.e. 16 h) with the drugs was used to maximise the degree of distribution and subsequent uptake within the TS. As shown in Fig. 1b, the TS demonstrated a high level of doxorubicin accumulation in the cells at the tissue surface. The degree of intracellular accumulation was considerably reduced deeper than the first few cell layers. An inner ring, consistent with the hypoxic zone (see Fig. 1a), was almost completely devoid of doxorubicin staining (Fig. 1b), reflecting poor intracellular accumulation in this area. The necrotic centre of the TS exhibited doxorubicin accumulation comparable with the outer cell layers and reflects the high levels of non-membrane-enclosed necrotic cell DNA available for doxorubicin intercalation and sequestration. A similar profile of accumulation was observed for 7-AAD, a protonatable amino derivative of actinomycin D (Fig. 1c). Overlap with the pimonidazole stain (Fig. 1a) reveals that the hypoxic region of viable cells did not accumulate 7-AAD to an appreciable level. The central region also displayed strong staining, due presumably to the ability of 7-AAD to interact with DNA that is readily accessible in these structurally compromised necrotic cells.

Figure 1d reveals the pattern of staining for BODIPY-Taxol in HT-29 TS of similar size and morphology. Unlike the situation observed for doxorubicin above, the distribution of BODIPY-Taxol was uniform throughout the TS and did not indicate any gradients of accumulation following this period of incubation. Clearly, the micro-environmental factors affecting the accumulation of these two compounds differ markedly and thereby impact on distribution through a solid tumour mass.

Effects of hypoxia on TS acidity and implications for drug distribution

In the large TS, as described earlier, it was clear that differential accumulation occurred in the normoxic/hypoxic zones for doxorubicin but not for BODIPY-taxol. Consequently, a simplified system was developed in order to model both zones independently and to elucidate the reason

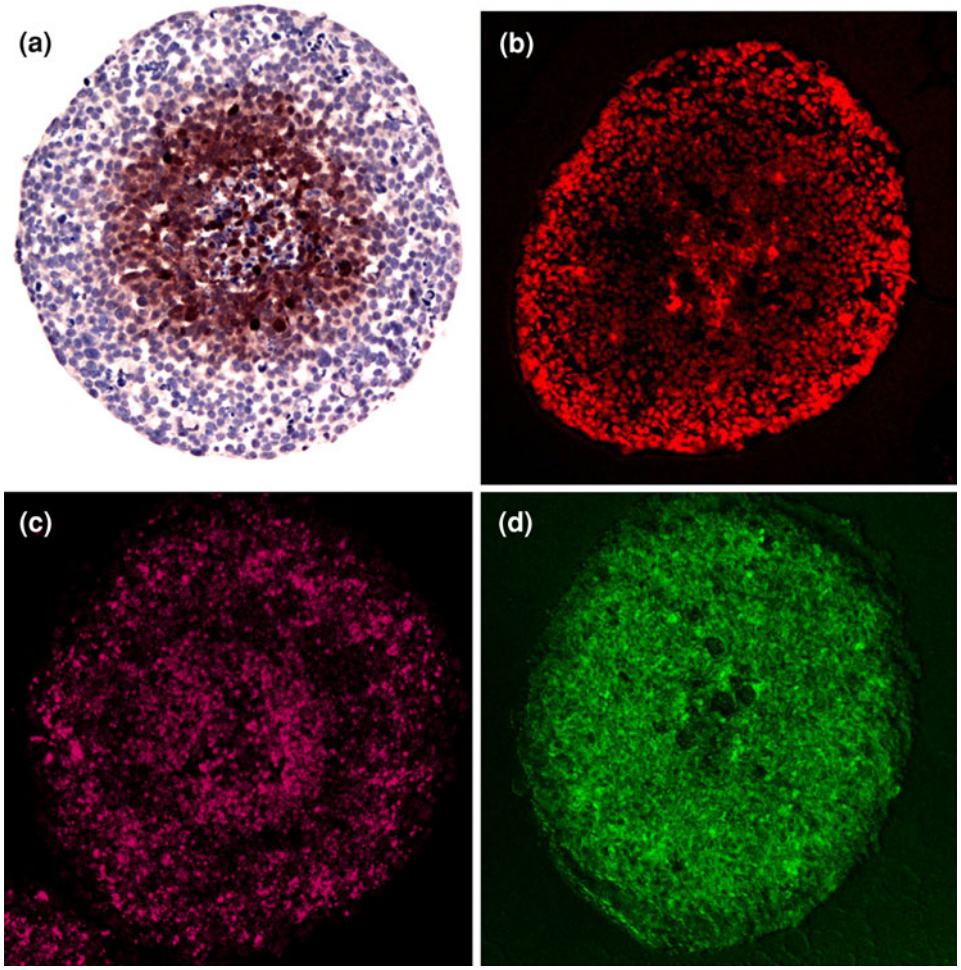

Fig. 1 Tumour spheroid hypoxia and drug accumulation. **a** Pimonidazole hydrochloride was added to TS comprising HT-29 cells grown to a diameter of approximately 500 μm. Immunohistochemical detection of pimonidazole adducts was made using sections from paraffin-embedded TS with the hypoxyprobe-1 monoclonal antibody, and the counter stain used was haematoxylin (*blue*). **b–d** TS from HT29 cells were grown to diameters of 500 μm and assessed for the distribution of **b** doxorubicin **c** 7-amino-actinomycin D and **d** BODIPY-Taxol by confocal fluorescence microscopy as described in the "Materials and methods" (colour figure online)

underlying the poor distribution of doxorubicin in deeper layers of the TS. Specifically, TS with diameters of ~200 μm were used in subsequent investigations since they do not show inherently hypoxic regions or the presence of a central necrotic core. In addition, the reduced diameter will enable drugs to rapidly achieve extensive penetration of the tissue. These smaller TS were examined under equilibrated normoxic (20% O_2, 5% CO_2) or forced hypoxic (0.1% O_2, 5% CO_2) conditions and the amount of drug accumulation quantified and compared.

Under normoxic conditions, the accumulation of doxorubicin in DLD-1 TS displayed dose dependence, with 44 ± 8 nmoles/mg protein accumulated in the presence of 100 μM doxorubicin. In contrast, the accumulation at the same concentration of doxorubicin was reduced fourfold to 10 ± 1 nmoles/mg protein when the TS were incubated under hypoxic conditions (Fig. 2a). This observation was repeated in HT-29 spheroids, where at the maximal doxorubicin concentration in the medium (i.e. 100 μM), the HT-29 TS accumulated 49 ± 4 nmoles/mg protein in normoxia, and this was reduced 2.5-fold (21 ± 1 nmoles/mg protein) during hypoxic incubation (Fig. 2b).

A normoxia/hypoxia accumulation difference was not observed with BODIPY-Taxol in TS comprising DLD-1 cells under the two levels of oxygen (Fig. 2c). At a BODIPY-Taxol concentration of 100nM, the TS accumulated 76 ± 6 pmol/mg protein under normoxia, which was not statistically different from the levels under hypoxia (91 ± 7 nmoles/mg protein). The lack of difference in accumulation of BODIPY-Taxol at two levels of oxygen is in agreement with observations with the drug in larger TS (Fig. 1d).

A potential explanation for the difference in accumulation profiles observed between doxorubicin and BODIPY-Taxol is the acidity that accompanies hypoxia in tumours. Doxorubicin is a basic compound with pKa = 8.2, and therefore, its ionisation state is sensitive to pH fluctuations in the physiological range. Tumour acidity is a product of many processes, and two of the major sources include CO_2 and lactic acid produced by bioenergetic metabolism of glucose and other fuels. Acidic components or precursors are expelled from cancer cells and consequently lead to a drop in the pH of the interstitial fluid and the solution surrounding a tumour. Consequently, the pH of the bathing

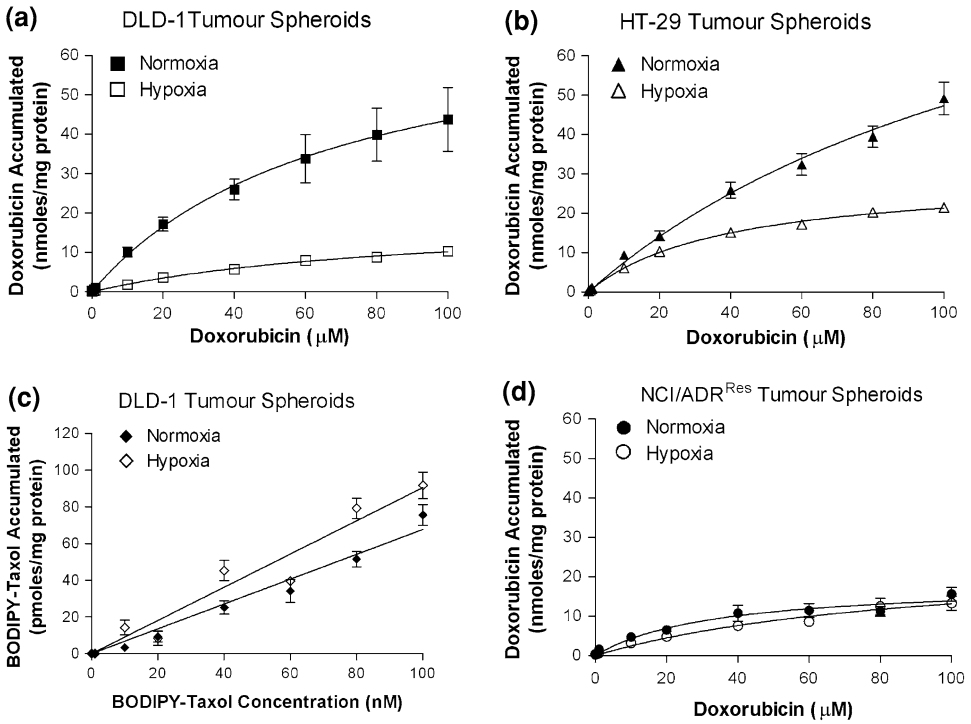

Fig. 2 Effect of TS exposure to hypoxia on accumulation of doxorubicin. Doxorubicin accumulation was measured in TS that had been exposed to normoxic or hypoxic conditions as described in the methods. TS were generated from **a** DLD-1, **b** HT-29 and **d** NCI/ADR^{Res} cell lines and incubated in the presence of 10 μM doxorubicin. Following incubation, the TS were solubilised in SDS and the amount of doxorubicin retained was measured by fluorescence spectroscopy using a standard curve with known drug concentrations. **c** shows the accumulation of the paclitaxel analogue BODIPY-Taxol (50nM) in TS comprising DLD-1 cells. The amount of BODIPY-Taxol accumulated within the TS was estimated using fluorescence spectroscopy with a standard curve of known drug concentrations. Values correspond to mean ± SEM from at least four independent observations and the data fitted using non-linear regression of the Langmuir Isotherm

medium was measured for TS comprising DLD-1, HT-29 and NCI/ADR^{Res} cell lines. Under normoxic growth conditions, the pH range of the medium bathing the TS was 7.25–7.30, but this was dramatically altered in hypoxia (Fig. 3). No such acidification was observed in bathing medium in the absence of TS (data not shown). Across the three cell lines, the pH of the bathing medium dropped to below 7.0, indicating a considerable acidification of the TS. The bathing medium of TS contains considerable buffering capacity, yet this was overwhelmed with a change of approximately 0.3 pH units due to the release of acidic compounds from the TS.

The influence of P-glycoprotein on drug accumulation in normoxia and hypoxia

Another common feature of solid tumours is the presence of the multidrug efflux pump P-gp. Expression of this transporter can greatly influence the cellular accumulation of chemotherapeutics including anthracyclines [46, 47]. The accumulation of doxorubicin was therefore also examined in TS comprising NCI/ADR^{Res} cells, which

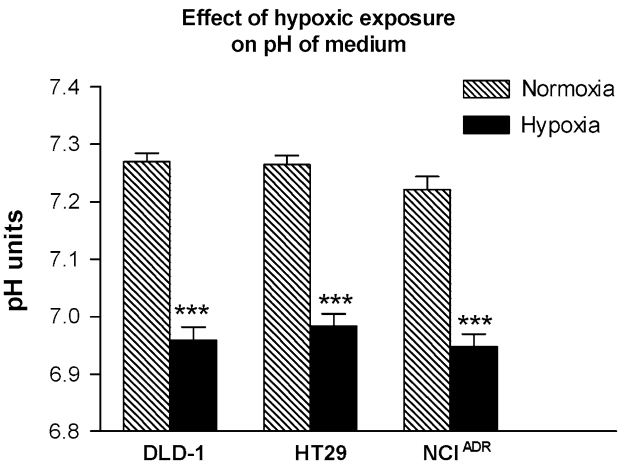

Fig. 3 Effect of TS exposure to hypoxia on pH of the medium. TS from several cancer cell lines were grown to diameters of 200–400 μm and exposed to a hypoxic environment for 16 h or incubated under standard conditions (i.e. normoxia). Immediately following the incubation, the pH of the bathing medium was measured. The histogram shows the mean ± SEM obtained from a minimum of four independent observations, and the symbol '*' indicates a statistically significant ($P < 0.05$) difference between hypoxic and normoxic growth conditions

over-express P-gp. Accumulation was measured under normoxic and hypoxic conditions in these TS to assess whether acidic pH associated with hypoxia could reduce doxorubicin accumulation on a background of already high P-gp-mediated exclusion. Figure 2d demonstrates that the accumulation profiles for doxorubicin under normoxia and hypoxia were almost identical. For example, at a doxorubicin concentration of 100 μM, the drug accumulation level was 16 ± 2 nmoles/mg protein in normoxia and 13 ± 2 nmoles/mg protein in hypoxia. Moreover, under normoxic conditions, the amount of drug accumulated was considerably lower compared with the levels observed for DLD-1 and HT-29 TS. This data suggest that when P-gp expression and associated doxorubicin efflux are high, exposure to hypoxia and acidity has minimal further impact on doxorubicin accumulation.

Does inhibition of P-glycoprotein improve drug distribution in TS?

The influence of P-gp on doxorubicin accumulation was further examined in hypoxic conditions in TS comprising the three cell types (Fig. 4). This was achieved by specific inhibition of P-gp by the modulator Tariquidar (XR9576), whose efficacy in TS has previously been demonstrated. Addition of Tariquidar to hypoxic DLD-1 TS (Fig. 4a) increased the amount of doxorubicin accumulated from 10 ± 1 to 17 ± 1 nmoles/mg protein, with a 500 nM dose to the bathing medium. This increase, whilst statistically significant ($P < 0.01$), failed to reach the levels obtained in DLD-1 TS incubated in normoxic conditions (44 ± 8 nmoles/mg protein). A similar situation was observed with the HT-29 TS (Fig. 4b), where the accumulation (doxorubicin dose 100 μM) was increased, albeit to a lower degree, from 21 ± 1 to 27 ± 2 nmoles/mg protein. Once again the increase fell considerably short of the accumulation level in TS under normoxic conditions (49 ± 4 nmoles/mg protein). Both HT-29 and DLD-1 cells display low-level P-gp expression [20–22], which is elevated by hypoxia-induced factors. The ability of Tariquidar to produce a small, but significant, elevation in the accumulation of doxorubicin indicates that this drug efflux pump plays a role in drug distribution within TS. The addition of Tariquidar to normoxic DLD-1 or HT29 TS failed to result in a significant elevation in doxorubicin accumulation (data not shown) indicating that P-gp is not the major determinant of doxorubicin accumulation in these TS under normoxia.

The established high-level expression of P-gp in NCI/ADR^{Res} TS, regardless of oxygen status, provided more conclusive information on its relative role in shaping doxorubicin accumulation (Fig. 4c). Under normoxic conditions, the accumulation of doxorubicin in NCI/ADR^{Res} TS was predictably low compared with those comprised of

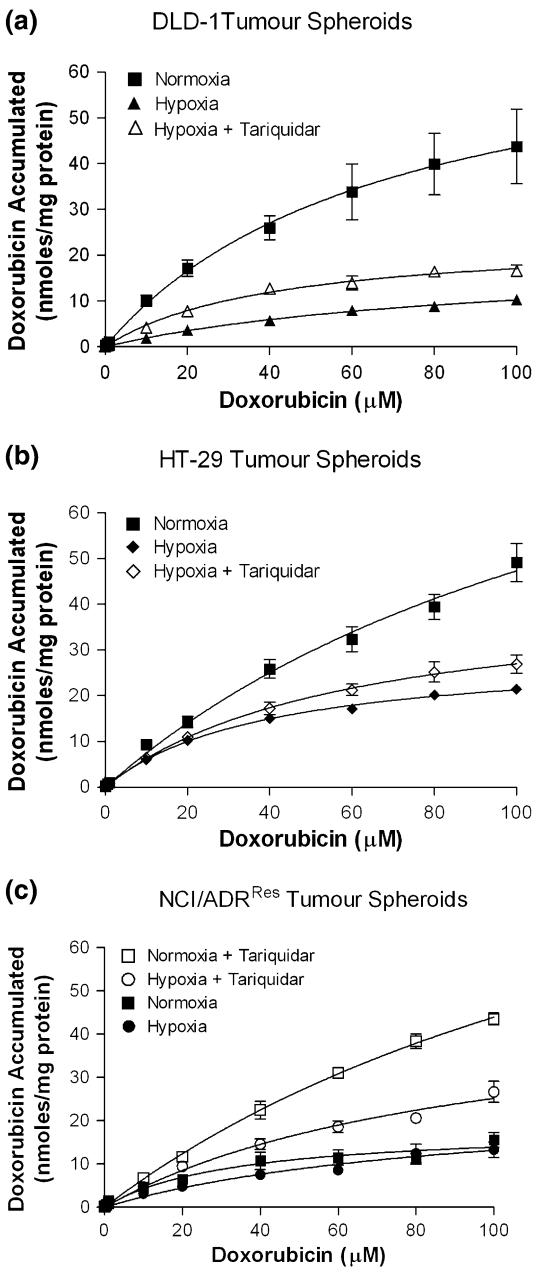

Fig. 4 Drug accumulation in TS following inhibition of P-glycoprotein. Doxorubicin accumulation was measured in TS that had been exposed to normoxic or hypoxic conditions as described in the methods. In **a**, **b**, TS grown in hypoxic conditions also contained the P-gp inhibitor Tariquidar (1 μM). In **c**, the presence of Tariquidar (1 μM) was included under both normoxic and hypoxic conditions. Following incubation, the TS were solubilised in SDS and the amount of doxorubicin retained was measured by fluorescence spectroscopy. Values correspond to mean ± SEM from at least four independent observations and the data fitted using non-linear regression of the Langmuir Isotherm

DLD-1 or HT29 cells. However, doxorubicin accumulation was increased 2.7-fold by the addition of Tariquidar (from 16 ± 2 to 43 ± 1 nmoles/mg protein), indicative of P-gp having a fundamental role in determining doxorubicin

distribution and uptake into TS cells. Addition of Tariquidar under hypoxic conditions also produced a significant increase of 2.1-fold from 13 ± 2 to 27 ± 2 nmoles/mg protein. Although the increase did not restore accumulation to levels observed in normoxic TS, it did reveal a considerable influence of P-gp expression on doxorubicin accumulation in hypoxia. Consequently, in TS that highly express P-gp, the accumulation of protonatable compounds such as doxorubicin is determined by both the presence of drug efflux pumps and the intra-tumoral acidification, but the impact of acidity may only be realised when P-gp has been inhibited. Both of these factors are directly related to the micro-environmental impact of a hypoxia.

A radial outgrowth assay was utilised to determine whether the alterations in drug accumulation do in fact impact on doxorubicin efficacy in TS. Table 1 demonstrates that hypoxic incubation (in the absence of drug) did not influence radial outgrowth from the TS comprising DLD-1, HT-29 or NCI/ADR^{Res} cells. Hypoxia did not influence the IC_{50} values for doxorubicin cytotoxicity in DLD-1 or HT-29 cells. However, the lower net doxorubicin exposure of cells in HT-29 and DLD-1 TS in hypoxia due to the acidic conditions when dosed with equivalent concentrations must be taken into account when considering these IC_{50} values. Therefore, taken together, this suggests that hypoxic cells may be more sensitive to doxorubicin if an equivalent normoxic exposure level could be reached.

The addition of Tariquidar to the DLD-1 and HT-29 TS did significantly increase the potency of doxorubicin to impair radial outgrowth, under both hypoxia and normoxia. This ability to restore chemosensitivity indicates the presence of P-gp in these TS types. It is important to recall that the reporter assay used in this investigation targets growth from the outer cells of TS (i.e. radial outgrowth from the TS surface) and therefore does not provide information on the deeper cell layers. In addition, the outgrowth assay required long incubation following the hypoxic incubation, during which cells (particularly outer layers) are likely to undergo reversion from the hypoxic phenotype and potentially under-estimate the influence of hypoxia on efficacy.

It was not possible to assign an IC_{50} value for doxorubicin in P-gp over-expressing NCI/ADR^{Res} TS since, due to these TS being highly doxorubicin resistant, a sufficient reduction in radial outgrowth was not achieved at the highest concentrations used. However, in the presence of Tariquidar, doxorubicin was capable of significantly impairing radial outgrowth from these TS. Interestingly, the potency of doxorubicin (in the presence of Tariquidar) was almost threefold lower in TS that were exposed to hypoxia. This suggests that for NCI/ADR^{Res} cells, hypoxia is in fact a significant contributor to the resistance of TS to doxorubicin. Moreover, the data suggest that in these cells, the two pathways may act in a synergistic manner.

Discussion

Doxorubicin accumulation and distribution within large tumour spheroids was demonstrated to show considerable heterogeneity. In contrast, the distribution of the taxane derivative BODIPY-Taxol was uniform in similar TS. The reason for this discrepancy was further investigated to reveal what factors underlie the aberrant distribution of doxorubicin. Micro-environmental factors may be responsible, given that the doxorubicin did not permeate hypoxic regions in the large TS. Hypoxic areas of TS, and avascular regions within tumours in vivo, are characterised by acidification, which is the likely environmental factor influencing accumulation of the basic drug doxorubicin [34, 43, 48, 49]. Another key factor in shaping the distribution profile of doxorubicin is the presence of the drug efflux

Table 1 The influence of hypoxia on the cytotoxicity of doxorubicin in tumour spheroids

Cell line	Condition	Outgrowth radius (d = μm)	Cytotoxicity doxorubicin (IC_{50} = μM)	Reversal Tariquidar (IC_{50} = μM)
DLD-1	Normoxia	100 ± 2	25.1 ± 3.7	7.7 ± 0.8*
	Hypoxia	92 ± 3	29.8 ± 4.2	7.0 ± 2.3*
HT-29	Normoxia	41 ± 1	10.3 ± 1.4	7.1 ± 1.6
	Hypoxia	42 ± 1	13.5 ± 3.5	9.3 ± 1.9
NCI/ADR^{Res}	Normoxia	74 ± 5	>100	5.6 ± 0.8
	Hypoxia	75 ± 2	>100	14.7 ± 1.5

Tumour spheroids were grown to a diameter of approximately 300 μm from DLD-1, HT-29 and NCI/ADR^{Res} cells. TS were grown under normoxic conditions or in a hypoxic environment for 24 h. The TS were then exposed to varying concentrations of doxorubicin (10^{-8}–10^{-4}M) for 16 h and then allowed to attach to tissue culture plates. The diameter (μm) of radial outgrowth was measured and plotted as a function of doxorubicin concentration. The potency of doxorubicin to hinder radial outgrowth was obtained using non-linear regression of the dose–response curve. TS exposure to doxorubicin was also assessed in the presence of Tariquidar (0.5 μM). The symbol '*' indicates a statistically significant ($P < 0.05$) difference in the IC_{50} value for doxorubicin in the presence of Tariquidar. All values (mean ± SEM) were obtained from at least four independent observations

pump P-gp [50, 51], which prevents intra-cellular accumulation and confers resistance to this drug. Hypoxia has been shown to increase the expression of P-gp in cancer cells and tumour spheroids [42, 52], in addition to endogenous sites of P-gp expression such as brain endothelial cells and hepatocytes [53, 54]. In addition, the presence of intra-tumoral acidosis has been associated with increased activity of P-gp [55–57] and a concomitant resistance of the tissue to chemotherapy. Consequently, the factors determining the accumulation and distribution of chemotherapeutic drugs within a tumour appear to operate in an additive or synergistic manner.

The schematic in Fig. 5 attempts to reconcile the interrelationship between acidity generated by bioenergetic metabolism and the presence of P-gp on cellular doxorubicin uptake. Uncharged doxorubicin (DOX) may rapidly cross plasma membranes along a concentration gradient; however, the presence of P-gp in the cell will avidly extract the drug from the lipid milieu and release it into the interstitial space. However, the 3-D arrangement of cells in solid tumours will generate a number of logistical problems (irrespective of P-gp expression) to the diffusion [21, 58, 59].

Figure 5 provides a summary of known pH homeostatic mechanisms in cancer cells and how our results fit into this. Oxidative and anaerobic pathways contribute to the production of lactic and carbonic acids [40, 60], thereby potentially reducing cellular pH. The presence of transporters and enzymes including MCT4 [66], NHE [61], CAIX [62, 63] and CAXII prevents acidification of the cytoplasm by expulsion of acidic molecules [38, 61]. Hypoxia has a multitude of effects on this homeostatic mechanism via altered protein expression patterns for the listed proteins [64] and through modified metabolism.

As dictated by the Warburg hypothesis [37], under anaerobic conditions, bioenergetic metabolism shifts to high reliance on cytosolic glycolysis to produce ATP [36, 65]. In order to maintain a high rate of glycolysis, pyruvate is almost exclusively converted to lactic acid, thereby regenerating sufficient levels of [NAD^+]. In order to prevent the accumulation of lactic acid in the cytosol, and hence acidification, it is effluxed via the monocarboxylate transporter MCT4 (for review see [66]). Recently, it has been suggested that tumour cells proximal to blood vessels (aerobic) express the MCT1 isoform, which mediates the uptake of lactate [67]. These aerobic cells will convert lactate to pyruvate, which is then subject to complete oxidation in the mitochondria. This metabolic interplay is further evidence of the adaptability of tumour cells to prosper in a hostile local micro-environment [4, 68].

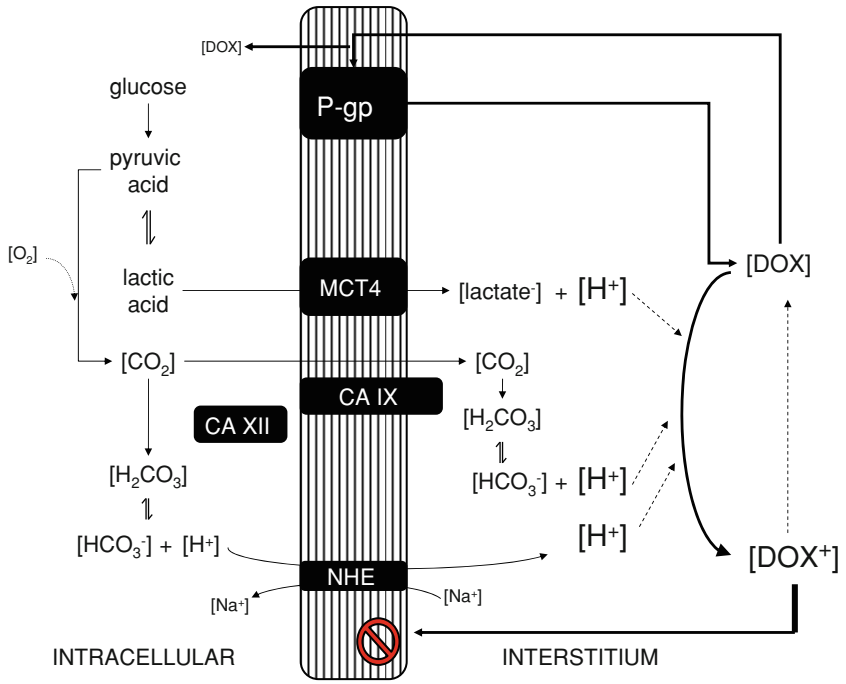

Fig. 5 Schematic outline of factors influencing doxorubicin accumulation. Non-ionised doxorubicin (DOX) may cross the plasma membrane of cancer cells by passive diffusion along a favourable concentration gradient. However, the presence of a drug efflux pump (P-gp) will significantly reduce the amount of DOX entering cells. DOX is in equilibrium between the non-ionised and ionised (DOX^+) forms, and the acidity of the interstitial space shifts the equilibrium towards the ionised and membrane impermeable form. The acidity is produced from aerobic and/or anaerobic metabolism of glucose. During aerobic respiration, the CO_2 produced will be converted to carbonic acid by cytosolic (CA) or extracellular facing (CA-IX) carbonic anhydrase isoforms. Lactic acid is another metabolic product associated with acidification. Consequently, this compound is extruded from cells by the monocarboxylate transporter (MCT4)

The acidification of interstitial pH will impair the accumulation of doxorubicin. The drug has a pKa = 8.2 and reduction in the pH from the physiological level will increase the proportion of ionised doxorubicin (DOX^+). The charged DOX^+ cannot readily cross the plasma membrane, thereby reducing the intracellular concentration and efficacy. The distribution profiles shown in this study highlight regions of TS in which cells accumulate doxorubicin. Compared with the cellular component of the TS, the volume of the interstitial space is very small. Minimal cellular accumulation of doxorubicin in the hypoxic zone resulted in a distribution profile, wherein the hypoxic regions of TS are devoid of doxorubicin.

Investigations with TS comprising cells that constitutively over-express P-gp revealed that inhibition of the protein could greatly increase the uptake of doxorubicin. However, TS that had been exposed to hypoxic incubation did not accumulate doxorubicin to the same extent as those in normoxia. The difference was attributed to the acidity produced by hypoxia and demonstrates that both mechanisms (efflux and ↓pH) are responsible for the reduced accumulation of drug. This is supported by a study demonstrating that inhibition of NHE (a contributor to pH regulation) results in increased doxorubicin accumulation [69]. The ability of Tariquidar to produce an increase in doxorubicin accumulation in hypoxic TS comprised of cells with low inherent P-gp expression indicates that expression of the pump is upregulated by hypoxic incubation as previously established [41, 42, 52, 55, 56].

Acidity of the interstitial space and the presence of P-gp in hypoxic regions of solid tumours therefore synergistically influence the distribution profile of doxorubicin and presumably other protonatable drugs. The other multidrug efflux pumps (ABCC1 and ABCG2) are also the known contributors to intra-tumour drug distribution and accumulation. Future studies will need to ascertain whether tumour micro-environmental factors also alter their expression in a similar manner to P-gp. Strategies aimed at improving chemotherapeutic efficacy will need to overcome both of pharmacokinetic barriers, namely drug transporters and tumour = microenvironment factors.

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