ABSTRACT

The failure of many anticancer drugs to control growth of solid cancers may stem in part from inadequate delivery to tumor regions distant from vasculature. Although the identification of new anticancer drug targets has led to the development of many new drug candidates, there is a lack of methodology for identifying drugs that adequately penetrate tumor tissue. We have developed a novel multilayered cell culture-based assay, which detects the penetration of anticancer drugs based on their effect within tissue. Drug exposures are made over 1 hour to one side of a disk of tissue ~150-μm thick, with the other side temporarily closed off, and penetration is then assessed 1–3 days later via bromodeoxyuridine-based detection of S-phase cells. Using this assay, the tissue distribution of a selection of anthracycline analogues was assessed. At clinically relevant exposures, none of the agents were able to affect cells on the far side of the culture at levels approaching that seen on the near (exposed) side. Doxorubicin and epirubicin exhibited ~10-fold decreases in the drug exposure seen by the cells on the far side relative to those on the near side of the cultures, whereas for daunorubicin and mitoxantrone, ~30-fold and >30-fold decreases were observed respectively. Results were consistent with the observed gradients in drug-derived fluorescence of doxorubicin, epirubicin, and daunorubicin. This model could be applied as a simple anticancer drug development screen to discover drugs that exhibit desirable penetration properties.

INTRODUCTION

In contrast to most normal tissues, with relatively high microvessel densities, the extravascular compartment of solid tumors often poses a significant barrier to the penetration of molecules supplied by the blood. Deregulated proliferation of tumor cells causes increased separation of blood vessels (1, 2) and unstable perfusion (3–6), which in turn leads to a reduction in the ability of molecules supplied from the blood to reach all cells within the tissue (7, 8). Tumor cells can be located up to 15–20 cells away from the nearest blood vessel (more in some cases), whereas in most normal tissue, cells are within a few cell layers of a vessel. Little is known of the ability of many of the most commonly used anticancer drugs to distribute within solid tumors (9). Measurement of gross tumor drug accumulation is a routine component of new drug development but tells little about extravascular drug distribution, especially in the case of drugs that are highly reactive in tissue. In principle, visualization of drug using radiolabeled or immunohistochemical-based assays is the most direct way to evaluate drug penetration. However, this approach is not always possible, and the detection of drug gradients in tissue does not necessarily indicate that the drug is ineffective in regions distant from blood vessels.

Advances in drug development have produced many new anticancer drug candidates; however, there are few tools available to select from these candidates—drugs that have the ability to adequately penetrate the tumor extravascular compartment. Drug-cell interactions within the extravascular compartment such as metabolism, binding, sequesterization, and uptake may all act to thwart the uniform distribution of a drug. We have assessed a novel in vitro assay to evaluate drug penetration that is based on detection of a drug’s effect in multilayered cell culture (MCC). MCCs consist of discs of tumor tissue, typically ~150-μm thick, grown from cells seeded on a permeable membrane (10, 11). They are similar to multicellular spheroids (12), but their planar nature permits flux through the cultures to be more easily measured (13, 14). Similar to spheroids, they exhibit a gradient in proliferation, but because MCCs grow as discs, this gradient forms as a mirror image from either side toward the middle.

In this study, we exploit this symmetry by exposing MCCs to drugs from one side and then comparing their effect on the cells located on the near (exposed) side versus the far side of the cultures. To model tumors in situ, drug exposures were made with the far side of the cultures temporarily closed off to media, thereby permitting drug to build up in the cultures. We have previously shown that proliferation can be maintained on the far side of ~175-μm thick MCCs during a 1-hour exposure preceded by a 45-minute equilibration period (15).

For this study, we selected the anthracyclines, doxorubicin (DOX), epirubicin (EPI), daunorubicin (DAU), and the related compound mitoxantrone (MIT), because of their similar mechanism of action but differing physicochemical properties and clinical activities. In addition, the first three agents fluorence and hence allow for direct comparison of drug effect with drug fluorescence in tissue. The agents are weak bases with pKa values of ~8.3 for DOX, DAU, and MIT and ~8.1 for EPI (16, 17) and at pH 7.4 are between 85 and 90% charged. Entry into cells is believed to occur via passive uptake of the neutral species for all four drugs. Of the three, DAU is most lipophilic, entering cells 3 to 10 times more quickly than DOX (18, 19), whereas EPI exhibits an uptake rate approximately twice that of DOX (20). Log octanol-water partition coefficients at pH 7.4 are DOX ~0.3, EPI ~0.6, DAU ~0.9, and MTX ~0.8 (21–23). All bind readily to DNA and accumulate in cells (24). From previous experience with these drugs, we anticipate that the more rapid rate of cellular uptake of the more lipophilic drugs may compromise their ability to penetrate into tissue.

MATERIALS AND METHODS

Monolayer Culture. HCT-116 human colorectal carcinoma cells were purchased from American Type Culture Collection. Cells were grown in monolayers using MEM (Life Technologies, Inc., Burlington, Ontario, Canada) supplemented with 10% fetal bovine serum (Life Technologies, Inc.) and passaged every 3 to 4 days.

MCC. Standard tissue culture inserts (CM 12 mm, pore size 0.1 μm; Millipore, Nepean, Ontario, Canada) were coated with 150 μL of collagen (rat tail type I; Sigma Chemical), dissolved in 0.01 mol/L HCl, and diluted 1:4 with 60% ethanol to 0.75 mg/ml and allowed to dry overnight. HCT-116 cells, 7.5 × 10^5 in 0.5 ml growth media, were then added to the inserts and incubated for 15 h to allow the cells to attach. The cultures were then incubated for 2 days in custom built growth vessels (Fig. 1A) to form MCCs ~150-μm in thickness. Each growth vessel contained a frame that held the inserts completely immersed in 130 ml of stirred media (700 rpm, 25-mm stir bar) under continual
drug from both sides but under different oxygenation and glucose conditions. Each MCC was exposed to a 7.5-mL stirred reservoir kept under controlled gassing and temperature. A silicone gassing and temperature. A silicone

side using a polyacrylate jig (Fig. 1B), designed such that the bottom of each insert was clamped against a polycrylate block, with a layer of Parafilm sandwiched in between to ensure a complete seal. MCC sides are defined as the near (exposed) and far (closed off). C, apparatus used during control experiments where MCCs were exposed to drug from both sides but under different oxygenation and glucose conditions.

**MCC Penetration Assay.** MCCs were exposed to anthracyclines from one side using a polyacrylate jig (Fig. 1B), designed such that the bottom of each insert was clamped against a flat block, with a layer of Parafilm (American National Can, Chicago, IL) sandwiched in between to ensure a complete seal. Each MCC was exposed to a 7.5-mL stirred reservoir kept under controlled gassing and temperature. A silicone o-ring was used to seal the gap between the MCC and the orifice in the reservoir. Once placed in the jig, MCCs were allowed to equilibrate for 45 minutes, and the drug was then added to the growth medium at concentrations of 0.3, 1, 3, and 10 μmol/L. After 1-hour drug exposure, the reservoirs were rinsed twice with fresh media. MCCs were then incubated for a second hour before removal from the jig to allow initial drug wash out from the topside only. MCCs were then removed, rinsed in fresh media three times for 15 seconds, and placed in a growth jar containing the two untreated control MCCs that were not subjected to closure. Separate control experiments were carried out to verify that closure of the MCCs did not, on its own, affect proliferation 1 or 3 days later at time of bromodeoxyuridine (BrdUrd) labeling. Media was replaced at 1 and 4 hours following return to the growth jar to reduce residual drug levels. Incubation with control MCCs allowed us to rule out the possibility that drug wash out into the media from the exposed sides of the MCCs was affecting results. MCCs were left for 1 or 3 days from the time of beginning drug exposure to allow manifestation of drug effect. After this, 100 μmol/L BrdUrd (Sigma Chemical, Oakville, Ontario, Canada) were added to the media, and cultures were incubated for 4 hours to label S-phase cells. MCCs were then removed, frozen in OCT medium (Tissue-TEK, Torrance, CA) and stored at −80°C until sectioning.

**MCC Control Assay.** Control experiments were carried out using a dual-reservoir apparatus (Fig. 1C) in which equal drug exposure was made to both sides of MCCs but under 20% oxygen, 1 g/L glucose in the first reservoir versus 0% oxygen, 0.1 g/L glucose in the second reservoir. Media were gassed overnight and then re-equilibrated for 1 hour upon insertion of the MCCs within the dual-reservoir apparatus. For these experiments, the media used was glucose-free DMEM (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Life Technologies, Inc.). Sodium bicarbonate levels were set to produce pH 7.4 under 5% carbon dioxide. Glucose was then added to the oxygenated side to match physiological levels, 1 g/L. On the anoxic side, the 0.1 g/L glucose were derived from the 10% fetal bovine serum. Drug exposure and wash out were carried out in the manner described in the previous section.

**BrdUrd Immunohistochemistry.** MCC cryosections, 10 μm, were air dried for 24 hours and then fixed in a 1:1 mixture of acetone-methanol for 10 minutes at room temperature. Slides were immediately transferred to distilled water for 10 minutes and then treated with 2 mol/L HCl at room temperature for 1 hour followed by neutralization for 5 minutes in 0.1 mol/L sodium borate. Slides were then washed in distilled water and transferred to a PBS bath. Subsequent steps were each followed by a 5-minute wash in PBS. BrdUrd incorporated into DNA was detected using a 1:200 dilution of monoclonal mouse anti-BrdUrd (clone BU33; Sigma Chemical) followed by 1:100 dilution of antimonium peroxidase conjugate antibody (Sigma Chemical) and 1:10 dilution of metal-enhanced 3,3′-diaminobenzidine substrate (Pierce, Rockford, IL). Slides were then counterstained with hematoxylin, dehydrated, and mounted using Permount (Fisher Scientific, Fair Lawn, NJ).

**Image Acquisition.** Using the NIH Image software application and user supplied algorithms, digital images of BrdUrd staining and drug fluorescence within MCC cryosections were analyzed in the following manner. Pixels making up the cryosection were first sorted based on their distance relative to either edge of the tissue. For BrdUrd images, the fraction of positively stained pixels at each position relative to the two edges was then calculated. Pixels 2.5 SDs above tissue background levels were classified as BrdUrd positive. For fluorescence images, the average value of pixels from each group relative to the two edges was calculated. Detection of tissue edges was facilitated by capturing high-contrast, bright-field images of the MCCs (before staining and mounting the slides in the case of the BrdUrd sections).

**RESULTS**

**Effect-Based Evaluation of Anthracycline Penetration in MCCs.** The penetration of the four anthracyclines was evaluated over a range of concentrations via detection of their effect on S-phase labeling using HCT-116 MCCs grown under controlled conditions in specialized growth vessels (Fig. 1A). Drug exposures were made to one side of the MCCs using the apparatus shown in Fig. 1B; the two sides of the MCCs were defined as the near side (exposed) and far side (closed off). Exposure from one side enabled a comparison of drug effect on cells that display similar rates of proliferation that are either directly in contact with the drug in media or separated from the drug by the thickness of the culture itself. After 1-hour drug exposures, cultures were returned to their growth vessels and incubated for 1 to 3 days in fresh media to allow manifestation of drug effect within the tissue.

Data from experiments where MCCs were exposed for 1 hour to 0.3, 1, 3, and 10 μmol/L EPI and then incubated for 1 day to allow time for manifestation of drug effect are shown in Fig. 2. Results show a gradual increase in depth of the effect of EPI from 0.3 to 10 μmol/L. Only at the 10-μmol/L exposure is EPI found to exert an equal effect.

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1 Internet address: http://rsb.info.nih.gov/nih-image/
to either edge of the MCC. The MCC exposed to 10 μmol/L EPI is approximately the same thickness it was at the time of treatment while the other cultures exposed to lower concentrations have grown increasingly thicker during the day after treatment.

Fig. 3 shows a summary graph comparing the effect of the four drugs on the near and the far side of MCCs 1 day after a 1-hour drug exposure. BrdUrd labeling in the first 30 μm of tissue on either side of the MCCs is expressed as the fraction of labeling seen on the respective sides of untreated MCCs from each experiment. Shaded regions on each panel indicate typical drug exposures achieved in humans (25–28). In all cases, the relevant human exposures are not high enough to achieve an equal effect on both sides of the cultures. For human exposures, typically 70 to 80% of the total drug will be serum bound (29, 30), whereas for the MCC work, carried out with 10% FBS, serum binding is estimated at 20 to 30% of total drug (29, 30). Hence, if data were plotted versus free drug, the estimated human exposures would likely be ~3-fold lower relative to the MCC data shown in Fig. 3. For DOX, EPI, and DAU, data from both near and far sides exhibit a general trend of decreasing labeling with increasing drug concentration. However, the MIT data shows an increase in labeling on the near side with increasing drug concentration. Because MIT already exhibits a large effect at the lowest concentration, this is likely due either to nonproliferation related BrdUrd labeling or to cell loss on the near side at the higher concentrations. From the data, estimates of the decrease in drug exposure seen by cells on the far side relative to the near side were ~12-fold for DOX, ~10-fold for EPI, ~30-fold for DAU, and >30-fold for MIT. These values were arrived at through determination of the drug concentration, which produced an effect on the near side that matched that seen at 10 μmol/L drug on the far side. The average fraction of BrdUrd-positive tissue in untreated MCCs was 0.40 ± 0.06 (SD) on near sides versus 0.46 ± 0.04 (SD) on far sides. This difference is thought to be mainly because of differences in edge detection at the tissue to media transition, near side, in comparison with the more well-defined tissue to membrane transition, far side. MCC data shown in Fig. 3 are expressed relative to untreated MCCs from respective experiments rather than the overall averages. Middle regions of the MCCs exhibited a significantly lower average BrdUrd-stained fraction [0.28 ± 0.05 (SD)].

Control experiments were carried out using a dual-reservoir apparatus (Fig. 1C) where 1-hour drug exposure was made to both sides of MCCs with normal conditions on one side versus low oxygen and glucose on the other (see Materials and Methods); results also shown in Fig. 3. The low oxygen and glucose levels were chosen to simulate levels seen on the far side of the MCCs during the closed-off experiments. Results were used to estimate what effect an equal drug exposure would have had on the two sides of the MCCs during the closed-off experiments. No significant difference in drug effect between the two sides was observed. These experiments were carried out separately from previous work and performed in different media (see Materials and Methods), which may explain their slightly lower BrdUrd-labeling values as compared with the other data presented in Fig. 3.

The effect of 1-hour drug treatment on MCC thickness 1 day after exposure is summarized in Fig. 4. Data are expressed relative to the average thickness of control MCCs at the time of exposure, 160 ± 20 μm (SD), as shown by the solid horizontal lines. Untreated cultures grown for 1 day from time of treatment reached 225 ± 30 μm (SD) in thickness, indicated by dashed horizontal lines. Drug-treated cultures are seen to lie between their starting thickness and that of untreated cultures left for the additional 24 h. MCCs exposed at the higher drug concentrations do not grow as thick, as expected from the reduction in cell proliferation. On the basis of this reduction in proliferation, cell loss due to removal of cells on the near side is likely no more than two to three cell layers. In separate experiments, MCCs

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Fig. 3. Comparison of the effect of the four anthracyclines on the first 30 μm of tissue on the near side (●) and far side (○) of MCCs 1 day after a 1-hour exposure using the apparatus shown in Fig. 1B: DOX (A), EPI (B), DAU (C), and MIT (D). Control experiments under conditions of normal (▼) versus low oxygen and glucose (▼), using the apparatus in Fig. 1C, are also shown. Data show BrdUrd immunostaining expressed as the fraction of control levels seen in untreated MCCs from the same experiments. Points show average ± SE (n = 4–6). Shaded boxes indicate typical clinical exposures. Differences in the effects of the drugs on the far sides of the cultures were found to be statistically indistinguishable, P > 0.05, except at the 10 μmol/L × hour exposure, P < 0.005 (ANOVA), where the effect of EPI was significantly greater than DOX.

Fig. 2. Distribution of S-phase cells in MCCs 1 day after a 1-hour exposure to EPI from one side (the top surface in the image). Untreated (A), 0.3 (B), 1 (C), 3 (D), and 10 (E) μmol/L EPI. One day after drug exposure cultures were exposed to 100 μmol/L BrdUrd for 4 hours from both sides to label S-phase cells. Cryosections counterstained with hematoxylin. Scale bars, 150 μm, show approximate thickness of control tissue at time of drug exposure.
exposed to 100 μmol/L DOX were only one to two cell layers thinner than MCCs exposed to 10 μmol/L DOX, indicating no significant change in cell loss.

MCCs cultured for 3 days after clinically relevant exposures showed a similar distribution of S-phase cells as seen after 1 day 3 μmol/L × hour DOX (Fig. 5A), 3 μmol/L × hour EPI (Fig. 5B), 1 μmol/L × hour DAU (Fig. 5C), and 1 μmol/L × hour MIT (Fig. 5D). Cultures continued to increase in thickness, and in all cases, there was an increase in proliferation toward the far sides indicating penetration limited drug effectiveness.

Fluorescence-Based Evaluation of Anthracycline Penetration in MCCs. The profile of anthracycline-derived fluorescence within cultures was examined using the closed MCC jig immediately after the end of a 1-hour drug exposure at 10 μmol/L and 1 day after incubation in fresh media. Only DOX, EPI, and DAU, which all possess the same fluorophore, could be examined. Emission intensities of the three drugs in aqueous solution were within 10% of each other. Fig. 6 shows average tissue fluorescence as a function of distance into the cultures from the near sides. Of the three drugs, DOX appears to accumulate the least over the 1-hour exposure with EPI and DAU reaching ~1.5 and ~7 times higher levels, respectively, on the near sides (Fig. 6A–C). The much higher accumulation of DAU in the tissue can be related to its higher partition coefficient, leading to a higher rate of entry in cells. Interestingly, the drugs show similar cellular accumulation from the middle regions toward the far sides of each culture, see Fig. 6D–F, which show the data on the same scale. Position within MCCs is presented as a fraction between 0 and 1 to allow comparison between MCCs taken immediately after drug exposure, which measured 140 ± 15 μm in thickness, and MCCs incubated for the additional day, which were 185 ± 20 μm in thickness.

A comparison of effect-based evaluation of drug distribution versus drug-derived fluorescence is shown in Table 1. Results from the two methods indicated that the effect-based data reasonably predicts drug distribution as seen from fluorescence data. Effect-based results were estimated from data in Fig. 3. The decrease in fluorescence on the far side relative to the near side was calculated by averaging fluorescence over the first 30 μm of tissue on each side of the MCCs shown in Fig. 6.

DISCUSSION

In this study, MCCs were used in a novel configuration to isolate the role of drug penetration from other factors that determine drug efficacy with depth into tissue. By temporarily closing off one side of the MCCs during drug exposure, a comparison was made between a drug’s effect on proliferating cells that were directly exposed to drug and those that lay on the far side of the MCCs. This technique differs from multicellular spheroid-based cell survival experiments, which predict overall drug toxicity with depth...
into tissue and combine changes in intrinsic sensitivity with penetration limited drug exposure (31, 32). Results showed that limited tissue penetration of the drugs resulted in a 10 to 30-fold difference in drug exposure between cells on the near versus far side of the cultures. None of the drugs were able to exert an equal effect on both sides of the cultures at their respective clinical exposures, indicating penetration-limited effectiveness. Surprisingly, in both the effect-based assay and direct fluorescence assay, the drugs performed similarly on the far sides of the MCCs regardless of their differing lipophilicity, and it was only on the near sides that their effects could be differentiated. Indicating that the increase in penetrative potential of the more lipophilic drugs was countered by their increased binding and sequesterization within cells.

Because of the sustained growth of the cultures after treatment, especially on the far side, it was not feasible to perform an in situ cell survival assay by leaving the cultures for longer periods. However, the distribution of BrdUrd labeling after 3 days was similar to that seen 1 hour after drug exposure, and results were generally consistent with published work with V79 multicellular spheroids, in which cell survival assays were performed. After DOX exposure of $\sim 3.5$ μmol/L × hour, spheroids that were incubated in drug-free medium for an additional 24 hour before disaggregation exhibited a surviving fraction of $\sim 0.2$ on the exposed outer layers versus $\sim 0.6$, 130 μm into the tissue (31). In the spheroid study, reduced intrinsic sensitivity of cells in the central area of the spheroids and time of disaggregation were shown to play key roles in determining drug toxicity. In comparison with spheroids, MCCs pose a greater barrier to penetration because of their planar rather than spherical geometry. In contrast, the outward-radial dilution experienced by a drug moving out of a blood vessel and into the surrounding tissue of a tumor likely poses an even greater barrier to penetration.

A main concern of closing off the MCCs during drug exposure was that deprivation of oxygen and glucose and build-up of lactate on the far (closed off) side could potentially lead to a change in the intrinsic sensitivity of the cells to the drugs. In this study, we simulated the effect of oxygen and nutrient deprivation on the far side of the cultures in control experiments where equal drug exposure was made to both sides of MCCs but with one side under normal oxygen and glucose and the other under low oxygen and glucose. Comparison of drug effect on the two sides was found to be within experimental error for all four drugs.

Direct fluorescence-based detection of the distribution of DOX, EPI, and DAU accumulation in MCCs immediately after exposure was consistent with results from the effect-based assay for drug penetration. The relative fluorescence on the near side of the MCCs matched the expected ranking of drug uptake rates from cell suspension data, determined mostly by lipophilicity and drug flu orescence on the far sides of the cultures, which was not significantly different for the three drugs. The effect of fluorescent metabolites of parent drugs was expected to play only a minor role on overall tissue fluorescence because of the short period of exposure. Drug fluorescence after 1 day of wash out was much reduced on the near edge of the cultures but still present toward the central region of the cultures.

In this study, it was found that despite their differing lipophilicity and rate of entry into cells, none of the drugs performed better either in transport or effect on the far sides of the cultures. Results indicated that the increase in diffusion potential of the more lipophilic drugs was tempered by the competing effects of more rapid DNA intercalation and other forms of sequesterization once in the cells, as indicated by the higher level of drug-derived fluorescence and degree of drug effect on the near sides. In the future, this assay could be used to balance these competing effects in the selection of new anthracycline analogues that possess improved penetrative characteristics.

### REFERENCES

Direct Assessment of Drug Penetration into Tissue Using a Novel Application of Three-Dimensional Cell Culture


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