# Penetration of Anticancer Drugs through Solid Tissue: A Factor That Limits the Effectiveness of Chemotherapy for Solid Tumors[1]

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## ABSTRACT

**Penetration of anticancer agents to cells distant from the vascular system is required for efficacy of cancer chemotherapy against solid tumors. Many solid tumors have a poorly formed blood vascular system with variable rates of blood flow and much larger intercapillary distances than those found in normal tissues. The requirement for drugs to penetrate several layers of tissue might pose a barrier to the effective treatment of solid tumors. Multicellular layers (~200 μm thick) were grown *in vitro* on Teflon membranes from EMT6 murine and MCF7 human tumors and have been used to quantitate the penetration of four widely used anticancer drugs through solid tissue. The penetration of doxorubicin and mitoxantrone was limited and very slow (<10% of the rate of penetration through the Teflon membrane alone). The penetration of methotrexate and 5-FU was more rapid (~30–50% of the rate of penetration through the Teflon membrane alone), but remains a substantial barrier to the effectiveness of these drugs. Strategies to improve the penetration of anticancer drugs through poorly vascularized tumor tissue have considerable potential to improve the outcome of chemotherapy for solid tumors.**

## INTRODUCTION

For the effective treatment of solid tumors, anticancer drugs must gain access to all viable cells within the tumors in sufficient concentrations to cause lethality. However, many solid tumors have a poorly formed blood vascular system with variable rates of blood flow and much larger intercapillary distances than those found in normal tissues (1, 2). The requirement for drugs to penetrate several layers of tissue might pose a barrier to the effective treatment of solid tumors. Moreover, the imperfect nature of vasculature in solid tumors also leads to tumor regions with deficiencies in the supply of oxygen and other nutrients and to the accumulation of metabolic acids (1, 3). Cells within these regions of tumors may be protected from conventional forms of cancer therapy; for example, there may be less uptake of weak bases such as doxorubicin or mitoxantrone into cells surrounded by an acidic microenvironment (4), and inhibition of cell proliferation in nutrient-deprived tumor regions may result in reduced cytotoxicity of these agents.

Until recently, the penetration of anticancer drugs into tissue has been studied by autoradiography or fluorescence microscopy after exposing multicellular spheroids to radiolabeled or fluorescent drugs (5–7). These studies have demonstrated poor penetration for doxorubicin, vinblastine, and methotrexate, with more uniform distribution for 5-FU (6–8). However, this method may be confounded by artifacts due to loss of drug during tissue processing. In an alternative method, the vital fluorescent dye Hoechst 33342 has been used to establish a gradient of drug concentration from the surface of spheroids and from blood vessels in murine tumors (9, 10). Separation of cells on the basis of Hoechst fluorescence by flow cytometry after treatment with anticancer drugs has allowed an estimation of cell killing as a function of depth in tissue. However, this method is complex and is dependent on microenvironmental factors that influence cell killing at different depths in tissue in addition to the compound's ability to penetrate tissue.

Recently, Cowan *et al.* (11) and Hicks *et al.* (12) developed an *in vitro* model that has allowed the direct quantitative assessment of the penetration of chemotherapeutic agents through a solid tissue environment. This simple method allows a drug to be introduced into medium on one side of a layer of solid tissue (MCL),[4] and its diffusion through the tissue is followed as a function of time by measurement of the appearance of the drug on the other side of the MCL (see Fig. 1). This method has been used to measure the penetration of radiosensitizing and experimental bioreductive agents (11), and a similar technique has demonstrated slow penetration of the anticancer agent paclitaxel (13). We report here studies of the penetration of four drugs commonly used in the treatment of solid tumors through MCLs derived from tumor cells of both murine and human origin.

## MATERIALS AND METHODS

**Cells.** Experiments were performed using the mouse mammary sarcoma cell line EMT6 and the human mammary carcinoma cell line MCF7. These cell lines were selected to

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[4] The abbreviations used are: MCL, multicellular layer; FU, fluorouracil.

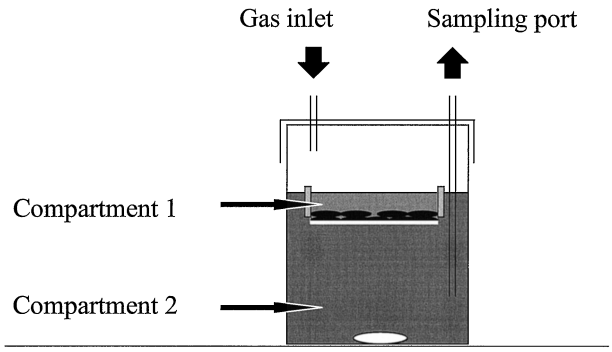


*Fig. 1* Schematic diagram of a multicellular layer developed by Cowan *et al.* (11). Drugs at selected concentrations were added to compartment 1. Samples were withdrawn through the sampling port from compartment 2. Vials were gassed with a mixture of 95% air and 5% $CO_2$.

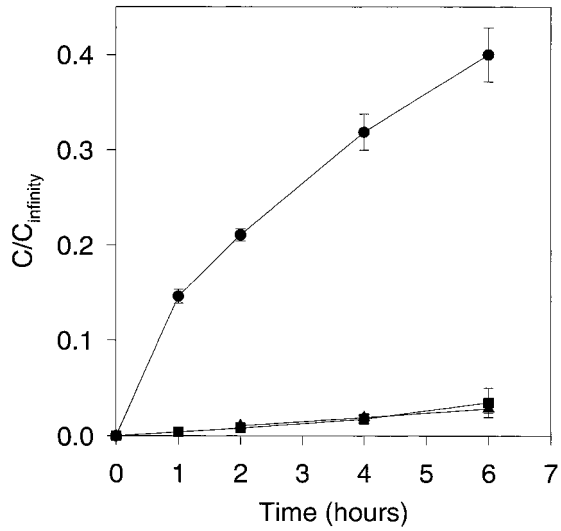


*Fig. 2* Penetration of [$^{14}$C]doxorubicin as a function of time. ●, ■, and ▲ represent the concentration ($C$) in compartment 2 (as a ratio of the expected concentration at equilibrium, $C_{infinity}$) for cell-free membranes and EMT6 and MCF7 cell layers, respectively. Five μg/ml (1 μCi) [$^{14}$C]doxorubicin was added to compartment 1. The doxorubicin radioactivity in compartment 2 was measured using a gamma counter. Data points represent the means of three or more experiments; *bars*, SE.

represent two species; they were also selected because their properties allow reproducible growth of MCLs with uniform thickness. The EMT6 cell line was obtained originally from Dr. R. Sutherland (Rochester, NY), and the MCF7 cells were purchased from the American Type Culture Collection (Rockville, MD). The cell lines were maintained in α-MEM containing 10% FCS at 37°C in a humidified atmosphere of 95% air plus 5% $CO_2$. Cultures were reestablished from frozen stock after ~3 months. Cell lines were tested routinely and found to be free of *Mycoplasma*. All experiments were performed using exponentially growing cells.

**Chemicals.** [$^{14}$C]doxorubicin and [$^{3}$H]methotrexate were obtained from Amersham Life Sciences (Buckinghamshire, England). 6-[$^{3}$H]5-FU and [$^{14}$C]sucrose were purchased from DuPont NEN (Billirica, MA). Mitoxantrone was obtained from Sigma (St. Louis, MO).

**Penetration of Anticancer Agents.** The *in vitro* model developed by Cowan *et al.* (11) and Hicks *et al.* (12) was used to measure drug penetration in a tumor-like environment. Briefly, this model involves seeding $2 \times 10^5$ exponentially growing cells on collagen-coated microporous Teflon membranes (Millipore, Bedford, MA). After the cells were allowed to attach for 4–24 h, the membranes were immersed in a large pool of stirred culture medium (~100 ml per membrane) to allow efficient nutrition from both sides. After 4 days of growth, the resulting structures were symmetrical multilayers of cells (MCLs), which developed a necrotic center surrounded by viable cell layers (11, 12).

Following the growth period, one randomly selected MCL was trypsinized to determine the total number of cells per MCL. The remaining inserts were used for the drug penetration studies. A schematic diagram of the experimental design is shown in Fig. 1 (see also Ref. 11). All drug solutions were mixed 1:1 with a 1% agar solution to prevent convective motion in compartment 1. Experiments using different agar concentrations in a cell-free system indicated that the agar presents no barrier to drug diffusion. The agent of interest was added to one side of the MCL (compartment 1) and the kinetics of its appearance on the opposite side (compartment 2) were determined by appropriate analytical methods. All experiments were performed at 37°C in an atmosphere of 95% air and 5% $CO_2$. Initial concentrations (in a volume of 0.5 ml) of 5 μg/ml (1 μCi) [$^{14}$C]doxorubicin, 100 ng/ml (10 μCi) 6-[$^{3}$H]5-FU, and 10 μg/ml unlabeled 5-FU, 10 μM [$^{3}$H]methotrexate, or 50 μM mitoxantrone were added to compartment 1. In these experiments, 3 μM of [$^{14}$C]sucrose was added to each drug solution (the sucrose is not taken up into cells and provides an internal standard) except in the doxorubicin studies. The penetration kinetics of [$^{14}$C]sucrose and the total number of cells were used to ensure minimal intra- and interexperimental variations of the MCL thickness. The flux of doxorubicin, methotrexate, FU, and sucrose through the MCL was assayed by measuring radioactivity in compartment 2 as a function of time of incubation. Mitoxantrone was assayed with HPLC using a Waters Radial-Pak reversed-phase $C_{18}$ column with an isocratic gradient of 73% ammonium formate and 27% acetonitrile. The flow rate was 1 ml/min, and detection was at 600 nm.

## RESULTS AND DISCUSSION

After a 4-day incubation, the number of cells that were obtained from MCLs derived from the MCF7 and EMT6 cells, respectively, were measured. MCLs with cell numbers that did not fall within the ranges ~1.5–2 $\times 10^6$ cells for MCF7 and 3.5–4 $\times 10^6$ cells for EMT6 were discarded. The penetration of sucrose through each MCL used in the experiments was measured as an internal standard for multilayer thickness. Consistent values for sucrose flux were observed in all experiments reported (data not shown).

The penetration of doxorubicin, mitoxantrone, methotrexate, and 5-FU as a function of time is shown in Figs. 2–5, respectively. The data are presented as the ratio of the concentration of drug observed in compartment 2 ($C$) to the drug concentration expected at equilibrium ($C_{infinity}$) as a function of

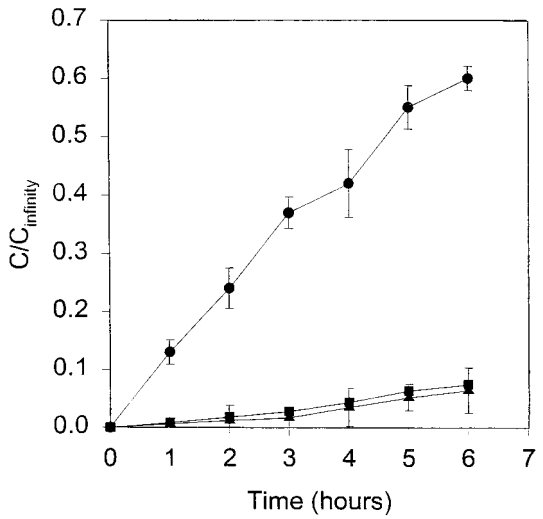


*Fig. 3* Penetration of mitoxantrone as a function of time. ●, ■, and ▲ represent the concentration (C) in compartment 2 (as a ratio of the expected concentration at equilibrium, $C_{infinity}$) for cell-free membranes and EMT6 and MCF7 cell layers, respectively. An initial concentration of 50 μM was used in compartment 1. Appearance of the compound in compartment 2 as a function of time was measured using HPLC. Data points represent the mean of at least three experiments; *bars*, standard errors of the mean.

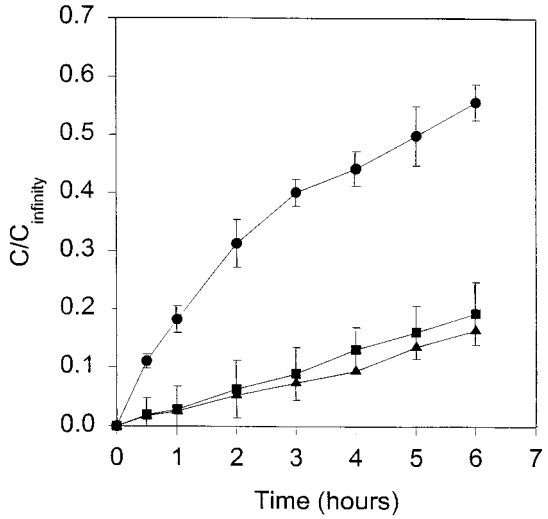


*Fig. 4* Penetration of [$^3$H]methotrexate as a function of time. ●, ■, and ▲ represent the concentration (C) in compartment 2 (as a ratio of the expected concentration at equilibrium, $C_{infinity}$) for cell-free membranes and EMT6 and MCF7 cell layers, respectively. An initial concentration of 50 μM was used in compartment 1. Appearance of the drug in compartment 2 as a function of time was measured using radioactive counting. Data points represent the mean of at least three experiments; *bars*, standard errors of the mean.

time. This value neglects any depletion of drug due to cellular uptake, but this is likely to be small given the limited number of cells per MCL. Penetration of each drug through a cell-free membrane was used as a negative control. The membrane itself provided an impediment to the diffusion of all drugs, and the

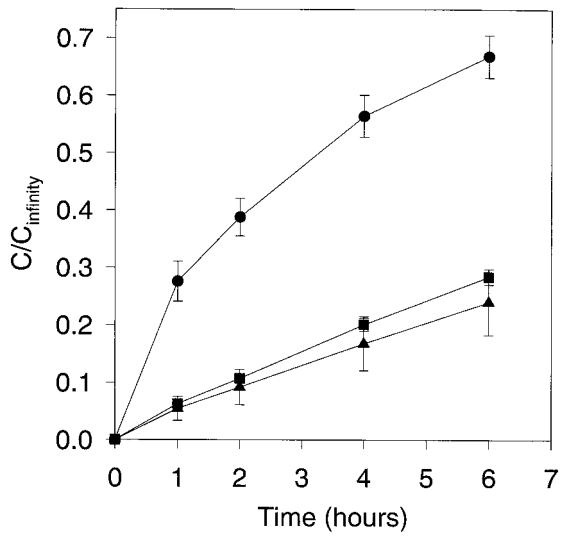


*Fig. 5* Penetration of [$^3$H]5-FU as a function of time. ●, ■, and ▲ represent the concentration (*C*) in compartment 2 (as a ratio of the expected concentration at equilibrium, $C_{infinity}$) for cell-free membranes and EMT6 and MCF7 cell layers, respectively. One hundred ng/ml (10 μCi) [$^3$H]5-FU and 10 μg/ml cold 5-FU were added to compartment 1. The radioactivity in compartment 2 was measured at selected times of incubation. Data points represent the means of three or more experiments; *bars*, SEs.

approach to equilibrium conditions was slower for doxorubicin than for other drugs. This might represent slower penetration through the porous membrane or nonspecific binding to it.

The MCL imposes a large barrier to the flux of both doxorubicin and mitoxantrone, with drug penetration of only about 5–10% of that seen with the cell-free system (Figs. 2 and 3). For doxorubicin, these results are in agreement with previous studies that have suggested poor penetration in spheroids and experimental tumors, using fluorescence microscopy or diffusion of the DNA stain Hoechst 33342, which allows assessment of the clonogenic survival of cells from different microregions (7, 9). Both mitoxantrone and doxorubicin are weak bases, which may allow for the sequestration of high concentrations of the drugs in acidic endosomes within cells, as demonstrated by Hicks *et al.* (12) with another weak base. In addition, they are both intercalating agents and bind avidly to DNA. Thus, poor penetration is probably due largely to accumulation in the first cell layers. Support for this hypothesis is provided by additional experiments that showed improved penetration of these agents through tissue layers containing cells which express the drug export protein P-glycoprotein (14). Although cellular uptake is important for the antitumor activity of these drugs, inhibition of their sequestration in acidic endosomes has considerable potential to improve tissue penetration and, hence, availability of the drugs to interact with target molecules distant from the blood supply. Inhibitors of sequestration in endosomes would be expected to be more effective against tumors than normal tissues because the vascular system in normal tissue is better developed, and drug penetration is therefore likely to be more uniform.

The penetration of the weak acid methotrexate through MCLs is greater than doxorubicin or mitoxantrone, with diffu-

sion that is ~30% of that through the cell-free system. Under physiological conditions, methotrexate tends to be in the charged form and is taken up into cells largely by a folate transport mechanism (15). Unlike doxorubicin and mitoxantrone, it is not sequestered in acidic endosomes, but it may be "trapped" inside cells by polyglutamation (15). Inhibition of cellular uptake (*e.g.*, by competing folates) or of polyglutamation might increase tissue penetration, but at the probable expense of a decrease in cytotoxic activity against proximal cells.

The rate of penetration of 5-FU through MCLs is comparable to that of methotrexate, with a flux ~40% of that observed through the Teflon membrane alone. 5-FU is a small water-soluble molecule that is not likely to be sequestered in vesicles or endosomes, but the MCL still imposes a significant barrier to its diffusion.

In summary, we have investigated the penetration properties of four widely used anticancer agents. Although methotrexate and 5-FU penetrate through solid tissue better than mitoxantrone or doxorubicin, even these agents have quite slow penetration through solid tissue compared with the cell-free system. Our results will ultimately require confirmation in animal models, but they suggest that the effectiveness of chemotherapy for solid tumors could be improved by approaches to increase drug penetration through solid tissue.

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