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Review

Aspects of cytotoxic drug penetration, with particular reference to anthracyclines*

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Summary. Experimental data, particularly derived from tumour spheroids, indicate that drug penetration barriers may be an important determinant of cytotoxic drug efficacy, even in spheroids of only a few hundred microns in diameter. Clinically, tumour masses of this size would equate with those micrometastases which are the target of adjuvant chemotherapy in a wide range of tumour types. It is apparent, therefore, that even at this relatively early stage of the metastatic process, which ultimately proves to be fatal in many patients, measures aimed at improving drug penetration may prove to be crucial in improving the therapeutic efficacy of cytotoxic agents.

Introduction

The aim of cancer chemotherapy is to eradicate clinically manifest or microscopic metastases. There is increasing recognition of the fact that poor drug penetration into solid tumours may be an important aspect of cytotoxic drug resistance [16]. Diminished access of chemotherapeutic agents to clonogenic cells in tumour nodules may be related to altered vasculature or drug penetration barriers. Penetration, therefore, will be determined by the concentration of free drug in the tumour vascular compartment and subsequent transport barriers interposed between the cytotoxic agent and its site of pharmacological action.

The introduction of the mutlicellular tumour spheroid system has proved a useful in vitro model for studying dynamic aspects of drug penetration. The use of this model circumvents the problems of drug disposition in vivo, but general pharmacokinetic principles can still be applied to analyses of the data obtained. The purpose of this short review is to describe:

1. The mode of passage of a drug from its site of administration to its site of pharmacological action
2. The application of pharmacokinetic principles to drug penetration
3. The role of multicellular spheroids as in vitro models for drug penetration studies

Our laboratory has a specific interest in anthracyclic compounds and we will cite specific examples of general principles for adriamycin and its analogues, where possible.

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Passage of drug from its site of administration to its site of pharmacological action

Once a water soluble drug entres the circulation from its site of administration, it becomes rapidly and widely disseminated throughout the body, via the bloodstream. There will usually be a dynamic equilibrium between drug at binding sites, including sites of biological action, and drug in intracellular or interstitial fluid. Further equilibria exist between drug in these compartments and free drug in plasma water, implying an indirect relationship between measured plasma drug levels and drug concentration at receptor sites (Fig. 1).

Plasma protein binding

Depending on the physico-chemical properties of the cytotoxic agent, it will partition to a varying extent between blood cells, plasma protein and plasma water. Plasma protein binding has a number of consequences. It will determine the relative amount of free drug available for tumor interaction, provide a "store" or buffer stabilising sudden alterations in drug availability, and is a possible site of drug interaction regarding drug displacement phenomena.

Egress from the vascular compartment

There are a number of modes of egress for a cytotoxic drug from the circulation. If sufficiently lipid-soluble, it can diffuse through the capillary endothelial lining into the extracellular fluid, whereas small polar compounds can diffuse out through water-filled pores between capillary endothelial junctions. Specific active transport mechanisms exist for a few molecules, e. g. hormones and amino acids, and it is possible that cytotoxic drugs could compete for these systems.

Malignant tumours appear to derive their vascular supply from newly formed vessels and by incorporation of existing vessels from host tissues [13]. The anatomy of the tumour microangioarchitecture is altered, with thin-walled erterioles deficient in medial smooth muscle and adrenergic innervation [10]. No formal studies of drug diffusibility across tumour capillary endothelium have been undertaken, but it would appear anatomically, if not functionally, that they might present a less well defined drug barrier.

In a series of classical experiments, Goldacre and Sylven [5] demonstrated that the blood-borne dye, lissamine green, did not diffuse into central regions of solid murine tumours grown subcutaneously. Nervertheless, it was pos-

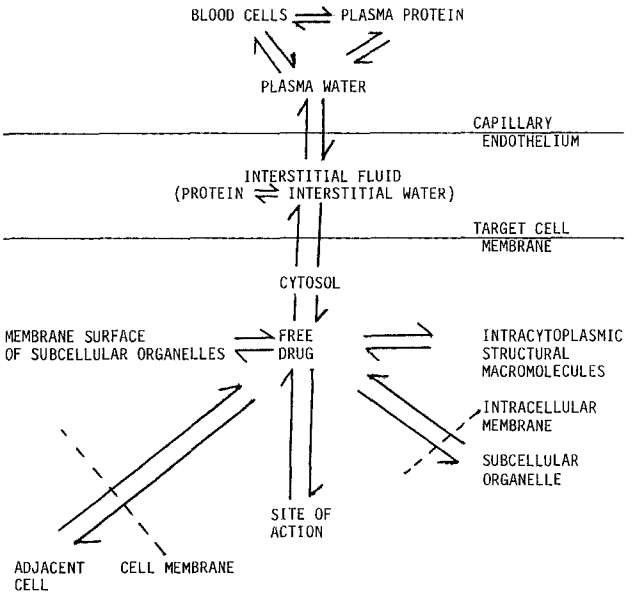

Fig. 1. Drug egress from the vascular compartment to its site of action

sible to regrow these tumours from tissue fragments transplanted from the dye-free regions. If the tumour microvasculature is compartmentalised in this way, it will lead to marked intratumoral differences in drug exposure depending on the ability of the drug in question to diffuse or be transported from the peripheral tumour vascular ring, in direct continuity with the systemic circulation, into the central region of solid tumours.

Diffusion through interstitial fluid

Within interstitial fluid, drug can partition between interstitial water and protein (consisting of structural proteins such as collagen and extravascular plasma proteins). It is possible that drug-cell surface interactions could occur with formation of ionic, Van der Waals or dipole-dipole bonds. The degree to which interstitial water will permeate throughout the tumour will depend on the compact "packing" of tumour cells and the tightness of intercellular junctions. This varies for different tumour types from simple membrane apposition to tight desmosomal junctions [18].

Transcellular drug transport

There are several potential mechanisms whereby a drug molecule might pass through cellular membranes to reach the cytoplasm. These include diffusion through water-filled membrane pores if the molecule is small and sufficiently polar to dissolve in water; diffusion of lipophilic drugs through the lipid domain of the cell membrane; and carrier-mediated transport systems which may be either passive (governed by drug concentration gradients) or active (coupled to energy expenditure). There is some evidence to suggest that adriamycin enters cells by diffusion of the electroneutral molecule through the lipid domain of the cell membrane [1]. The degree of passive diffusion is determined by the concentration gradient across the membrane, membrane thickness, total surface area of membrane exposed to drug, duration of drug-cell contact, and membrane permeability. As previously mentioned, the physico-chemical properties of the drug are also important, particularly charge and lipid solubility.

There is microheterogeneity of intratumoral pH, related to areas of necrosis and hypoxia [17]. Basic drugs such as adriamycin (PK_a 7.6–8.2) will tend to ionise at acidic pH and are thus limited in their ability to cross lipid membranes. The oil-water partition coefficient remains a useful marker of lipid solubility and will determine the ease with which the drug can traverse the lipid portion of the cell membrane. Adriamycin has the particular property of self-association to form dimeric structures through a π electron interaction of the tetracyclic anthraquinone rings [1]. Because of this phenomenon, the kinetics of cell uptake of adriamycin appear to fulfil the criteria for a carrier-mediated, saturable system. However, careful analysis of experimental data derived from Dalmark and Storm [1] favours the explanation that adriamycin is transported by diffusion of the monomer through the cell membrane.

Once in the cytoplasm, the drug may have to cross further intracellular membranes before reaching its site of pharmacological action. In common with other compartments through which the drug has diffused, there is a potential for reversible or irreversible binding to intracytoplasmic macromolecules, e. g. protein, RNA, and cytoplasmic membrane systems (Fig. 1).

Intercellular drug transport

Cell-cell transfer of active drug molecules may be accomplished by different means. Drug could be transported by specific transport or efflux systems, or by simple diffusion through the lipid domain of the membrane. The compactness of tumour cells and the number and type of intercellular connections would determine whether drug passes directly from cell to cell or is first transported into an intercellular space. Intercellular channels have been demonstrated by electron microscopy after staining with uranyl acetate [18], and this could represent a relatively rapid method of drug penetration to the centre of solid nodules (a "snakes and ladders" analogy would be appropriate).

Gap junctions are semipermeable intercellular connections which allow transfer of relatively small molecules (up to a molecular weight of around 1000), e. g. cyclic nucleotides [9]. In addition, the junctions have specific shape and charge characteristics. We have shown that gap junctions exist in culture between certain lines of human non-small cell lung tumour cells. It would seem possible that some cytotoxic drugs, particularly the antimetabolites, could use gap junctions as a rapid mode of intercellular transport.

The application of general pharmacokinetic principles to analysis of drug penetration

Advances in analytical techniques have allowed precise measurement of a wide range of cytotoxic drugs. Gel filtration, ultrafiltration and plasma dialysis allow determination of free drug, but usually whole plasma measurements are quoted. This, of course, gives only indirect evidence of the amount of drug at its site of action. It is possible, in certain cases, to measure simultaneous plasma and intratumoral drug concentrations and to relate these by means of pharmacokinetic models. Therefore it is possible to assess penetration in a crude, quantitative way. This approach will not, however, reveal the "pattern" of intratu-

moural drug distribution. By infusing high-dose adriamycin (10 mg over 2 h) via an intracarotid cannula in rats bearing subcutaneous tumours, we have shown by fluorescent microscopy that the anthracycline is located in the well-perfused periphery of the tumour, a situation similar to that seen with lissamine green [14]. As this drug would appear to be compartmentalised within the tumour, a measure of drug expressed per total tumour weight would only give an inaccurate "average" value, taking no account of intratumoral distribution.

The volume of distribution (Vd) of a drug may be defined as a proportionality constant which relates the plasma concentration to the total amount of drug present in the body. This parameter has no direct physiological meaning, but there are "ideal substance" which distribute into discrete compartments with physiological values. If a drug has extensive intracellular binding sites, then it will have a large Vd. The calcium channel blocker verapamil has been shown to enhance the cytotoxicity of adriamycin in vitro, probably by inhibiting active anthracycline efflux [7]. We have recently demonstrated that co-administration of verapamil and adriamycin to patients with limited-stage small cell lung cancer results in a significant pharmacokinetic interaction. There is an apparent increase in the peripheral compartmental Vd of adriamycin associated with administration of verapamil. Verapamil is a vasodilator and increases tumour blood flow by 50% in rats bearing a hind limb sarcoma [6]. Alterations in intracellular binding or vasodilation with increased peripheral tissue adriamycin delivery could explain the altered Vd.

Area under curve

We have already defined the factors which control passive diffusion of drug molecules. All mathematical expressions describing diffusion have terms including the concentration gradient and duration of drug exposure. The relationship between cytotoxic pharmacokinetic parameters and pharmacodynamic parameters of response have been previously discussed by Powis [15]. With regard to penetration by passive diffusion, it would seem most appropirate to relate this to the integral of concentration × time from zero to infinity, i. e. area under the curve (AUC) of the plasma concentration time curve. This expression would relate total tumour drug exposure, and would therefore seem possibly to be a better measure of drug penetration, than peak plasma drug levels.

However, this may not hold for all drugs. Intracellular levels of adriamycin and daunomycin in leukaemic cells have been measured after rapid (10 min) or prolonged (24 h) drug infusion [12]. Despite similarity in the plasma AUCs for rapid and prolonged infusions, the intracellular peak levels and AUCs were some 2–3 times higher after prolonged infusion. It would appear that for certain drugs, the temporal component assumes greater importance than the term denoting the concentration gradient in determining the degree of intracellular drug uptake by passive diffusion.

Tumour blood flow

There has been some interest in the study of tumour blood flow and its potential manipulation using a variety of vasoactive drugs. Reports have been made of increased, normal or decreased sensitivity to vasoactive drugs on the tumour microvascular bed [10]. The hypothesis underlying this work is that increasing tumour blood flow relative to normal tissue flow should provide enhanced delivery of chemotherapeutic drugs and perhaps increase tumour oxygenation. Organ clearance of a drug can be determined by a subtractive technique, if drug concentration measurements can be made in arterial and draining venous blood:

$$U = \frac{Q \cdot C_A - C_V}{C_A} = Q.E$$

where U = clearance, Q = blood flow, C_A = arterial drug concentration, C_V = venous drug concentration, E = extraction ratio.

If the extraction ratio is low, the tumour drug clearance will be relatively unaffected by alterations in blood flow. However, if the extraction ratio is high, then clearance will be proportional to blood flow. There are few data concerning tumour drug extraction ratios, but if they are low, then manoeuvres to increase tumour blood flow would have relatively little therapeutic benefit.

The multicellular spheroid as an in vitro model for studies on drug penetration

The multicellular spheroid model was developed as a three-dimensional system of intermediate complexity between monolayer and solid tumours grown in vivo, which would simulate the growth properties, cellular kinetics and microenvironment of a micrometastasis prior to vascularisation. It is therefore possible to study drug penetration and distribution within solid tumours free from the constraints associated with host animal drug distribution and anomalies of tumour vasculature.

A number of techniques have been devised to study the penetration of drugs into spheroids. In addition to conventional autoradiographic methods, which have been used successfully to demonstrate binding of methotrexate to dihydrofolate reductase in human osteosarcoma spheroids [19], Nederman et al. [11] have developed a "dry" method, which does not disturb the distribution of water-soluble substances, based on freeze-drying and vapour fixation of spheroids prior to preparation for autoradiography. They examined the penetration properties of a number of low-molecular-weight substances (such as ^{3}H-*D*-leucine and ^{3}H-thymidine) and the cytotoxic drugs ^{3}H-5-fluorouracil and ^{3}H-vinblastine. 5-Fluorouracil distributes rapidly and evenly through human glioma spheroids, whereas vinblastine is localised to the outer three to four cell layers. Poor drug access has been implicated in spheroid resistance to vincristine and the acridine derivative, *m*-AMSA [20, 21].

On the basis of flow cytometry and fluorescent microscopy, penetration barriers have been postulated for adriamycin in V79 Chinese hamster ovary spheroids [2, 16]. Intact spheroids are more resistant to treatment with cytotoxic drugs than are the component cells after disaggregation by trypsin. Part of this resistance may be attributed to penetration failure.

Utilising human non-small cell lung tumour spheroids, we have shown that the lipophilic anthracycline analogue, 4′-deoxydoxorubicin, penetrates further into spheroids than adriamycin, as assessed by fluorescent microscopy (Fig. 2). Clonogenic survival after monolayer exposure to the two drugs is very similar, whereas the lipophilic ana-

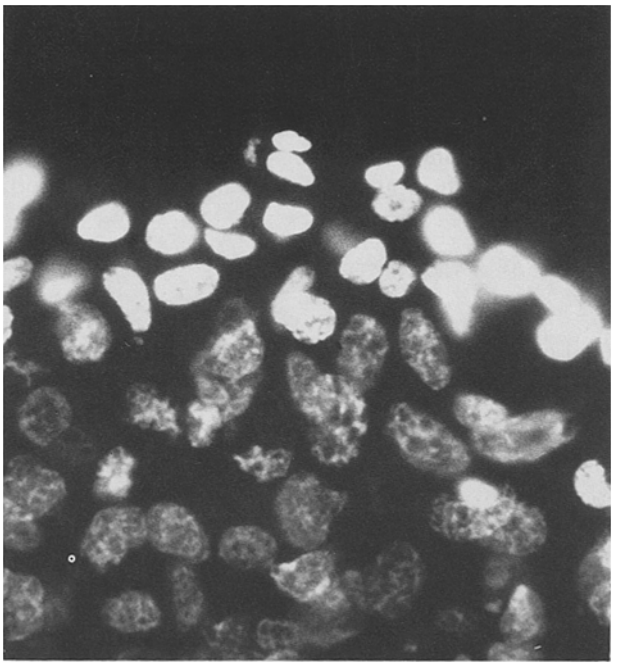

Fig. 2. Fluorescent photomicrograph showing the diffusion gradient for adriamycin from the external *(arrowed)* surface of a human lung tumour spheroid (magnification ×300)

logue induces a significantly longer growth delay and greater clonogenic cell kill after spheroid disaggregation than the parent compound. It would appear that drug penetration barries may contribute significantly to cytotoxic drug resistance and that these can be partly overcome by a lipophilic derivative.

Clinical relevance?

With the increasing trend towards combining animal pharmacokinetic data with preclinical toxicological studies in novel drug development programmes, it should soon be possible to demonstrate the relationship between administration schedules and parameters of response and toxicity. There already exist some human data, insofar as low-dose infusion of adriamycin seem to cause less cardiotoxicity and may be equally efficacioŭs regarding tumour response [8]. In addition, there appears to be enhancement of intracellular levels of adriamycin in circulatory leukaemic cells when infusion is prolonged to 24 h [12]. Clearly, more clinical trials varying the mode of administration of commonly used cytotoxic drugs are warranted.

We have demonstrated with our spheroid model that lipophilic analogues of adriamycin do appear to penetrate more deeply with associated improvement of the cytotoxic response. It may be possible to co-administer a "penetration-enhancing agent" which will increase drug uptake of central solid tumour cells. Our preliminary data indicate that simultaneous treatment of lung tumour spheroids with low doses of a polyoxethylated surfactant (Brij 30) tends to increase adriamycin penetration and increase response. There is, of course, the problem of general or non-specific enhancement of drug uptake, with the consequences of increased toxicity to myeloid or gastrointestinal cells. It may be possible to target drug to the tumour by using lipophilic vehicles, such as low-density lipoprotein (LDL), for which there are tumour receptors [4]. In a series of experiments with daunomycin coupled to LDL we have shown a similar degree of cytotoxic activity (relative to free daunomycin) in monolayer, but improved activity in the spheroid; therefore, it may be possible to improve drug tumour availability by targetting and by improving penetration.

There has been a considerable upsurge in interest in regional chemotherapy over the past several years due to the increasing availability of new technology that allows safe administration of chemotherapy and the growing understanding of the pharmacokinetic principles underlying rational usage of the technique. Intraperitoneal adriamycin and cis-platinum have been used for treatment of ovarian cancer, and it is possible that co-administration of surface-active agents or use of lipophilic anthracycline derivatives could increase drug penetration into tumour nodules without altering systemic drug exposure. It is of interest to note that after intravesical administration of similar doses of adriamycin and epirubicin (a lipophilic derivative), systemic levels of epriubicin are significantly higher than those of adriamycin [3]. Given the impermeant nature of the vesical epithelium it is possible that the lipophilic drug penetrates better through the bladder wall, and by inference intravesical tumour, than adriamycin.

The concept of adjuvant chemotherapy evolved from the idea that by the time of treatment for the primary tumour, by means of surgery or radiotherapy, most tumours have already established micrometastases. If, as was stated initially, the continuing hope for chemotherapy is complete eradication of all disease, and given the finding of significant drug penetration barries in tumour spheroids a few hundred microns in diameter, then it is apparent that failure of drug penetration does contribute significantly to drug resistance in the clinical setting.

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