Cancer Chemother Pharmacol (1988) 22: 21–25

Cancer Chemotherapy and Pharmacology

Penetration of substances into tumour tissue

Model studies using saccharides, thymidine and thymidine-5'-triphosphate in cellular spheroids*

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Summary. In order to achieve a better understanding of factors involved in drug penetration into poorly vascularized tumour tissue, the penetration of some model substances was studied in vitro. Multicellular human tumour spheroids were used as model system. The test substances were [^{3}H]thymidine and [^{14}C]glucose, both of which are capable of passing easily through cell membranes, and [^{3}H]thymidine-5'-triphosphate, [^{3}H]sucrose and [^{3}H]inulin, all of which are unable to pass directly through cell membranes. The penetration of these substances was studied using a dry histological and autoradiographical method preserving the distribution of water-soluble substances. The two thymidine compounds penetrated very efficiently into the spheroids, and their penetration patterns were rather similar. The saccharides differed somewhat in their penetration properties. Glucose had the fastest penetration and inulin the slowest. After 15 min, however, inulin was also found isotropically distributed within the spheroids. Thus, extracellular penetration seemed to be a possible way for a substance to reach the central parts of a spheroid. The differences between the saccharides could be due to some extent to differences in molecular weight and solubility.

Introduction

When chemical agents are used in tumour therapy, it is desirable that they reach all regions within the tumour. This can be a problem, since the rapid growth of certain solid tumours results in insufficient vascularization, leaving regions of viable cells distant from the blood capillaries [3, 20]. In previous studies in which multicellular spheroids were used as in vitro models for nodules of solid tumours [2] resistance to several drugs was found. The existence of drug penetration barriers has been suggested as being at least, partly responsible for such resistance to vinblastine [13, 14], adriamycin [8, 22] and methotrexate [24]. The penetration capacity of a substance into unvascularized tissue is probably determined by several factors, e.g. molecular weight, charge and polarity of the substance and such factors as the amount and composition of extracellular matrix surrounding the cells, and finally, by properties of the cells, e.g. the number of microvilli and the presence or absence of receptors. However, quantitative contribution of each factor is not known. It is hoped that studies on the penetration patterns in cellular spheroids will yield some information on this point.

In this work, our intention was to investigate whether substances with molecular weights in the same range as cytotoxic drugs can reach deeplying cells in tumour spheroids only by penetration through the extracellular matrix or whether they have to pass through cell membranes. The existence of an extracellular matrix in cellular spheroids has previously been demonstrated [18]. We used substances with known capacity to pass easily through cell membranes [thymidine (TdR) and glucose] and related substances [thymidine-5'-triphosphate (TTP), sucrose and inulin] lacking this capacity.

Materials and methods

Cell cultures. Three different, established human tumour cell lines were used, the glioma cell line U-118 MG [6, 19], the thyroid cancer cell line HTh-7 [6] and the osteosarcoma cell line U-393 OS [6], all established by Pontén, Westermark and co-workers at the Wallenberg Laboratory, Uppsala, Sweden. All these cell lines can be cultured both as monolayer cultures and as spheroids. They were chosen because their growth characteristics have previously been well described [6] and the spheroids have recently been used in a number of studies on, for example, oxygen measurements [1], radio- and chemosensitivity [4, 13], penetration properties [14, 16, 17] and extracellular matrix production [18]. It should be noted, however, that these spheroids were not used as models for their corresponding tumours in vivo, but merely as a model system for unvascularized nodules of any solid tumour.

Spheroids were cultured by the liquid overlay technique [5, 12, 26], i.e. by seeding suspended cells in an agarose-coated culture dish and allowing the cells to aggregate into small cell clusters. These aggregates continued to grow by proliferation. The culture medium used was Ham's F10 supplemented with 10% newborn bovine serum, L-glutamine (2 m*M*), penicillin (100 units/ml) and streptomycin (100 μg/ml) (Flow Laboratories Swedish, Stockholm, Sweden). During analysis all spheroids were in the range of 400–600 μm in diameter. This is the size range in which necrosis starts to develop [6].

* The work described in this paper was financed by the Swedish Cancer Society and the Swedish National Defence Research Institute

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Test substances. The following radioactively labelled substances were used: [methyl-^{3}H]thymidine ([^{3}H]TdR; 185 GBq/mmol), [methyl-^{3}H]thymidine-5'-triphosfate ([^{3}H]TTP; 1.1 TBq/mmol), L-[1-^{14}C]glucose (2.15 GBq/mmol), [6,6'(*n*)-^{3}H]sucrose (13 GBq/mmol) and [^{3}H]inulin (72.5 GBq/mmol). All test substances were purchased from the Radiochemical Centre, Amersham, England. For the penetration studies they were diluted to 1.0 MBq/ml (about 27 μCi/ml) in culture medium.

Penetration studies. The dry histological and autoradiographical method used has previously been described [15, 16]. This method preserved distribution in the tissue of the water-soluble fraction of tested substances [16]. Briefly, spheroids were allowed to attach to small cover slips in normal culture conditions overnight and were then incubated for an appropriate period (0.5, 1, 2 or 15 min) in medium containing the radioactive test substance. The spheroids were then quickly frozen (−196° C), freeze-dried at −80° C to −70° C (TIS-U-DRY, FTS Systems, Stone Ridge, NY, USA) and vapour-fixed in an 80° C paraformic aldehyde-saturated atmosphere. The fixed spheroids were wax-embedded after xylene infiltration, sectioned (5 μm), and dry-mounted on microscope slides. A slide with a section was put together with another slide covered with dry photoemulsion (Ilford K5). These glasses were tightly pressed together in a "sandwich" for 4 weeks of exposure. This contact autoradiography was used to avoid disturbances in the distribution of unbound test substance during the autoradiographic procedure. After developing (Kodak D 19) and fixing (Kodafix), contact autoradiograms were visually inspected in the microscope (Leitz Orthoplan) and photographed on Kodak Panatomic-X by a microscope camera (Leitz Vario-Orthomat). Evaluations were performed by counting grains at high magnification, in small distinct areas along tracks from outside the sections towards the central parts. This made it possible to obtain grain density in contact autoradiograms as a function of the depth inside spheroids.

Results

The two nucleosides TdR (mol. wt. 242) and TTP (mol. wt. 484) penetrated very rapidly into the U-118 MG (Fig. 1) and the U-393 OS (Fig. 2) spheroids. After 1 min, both substances were isotropically distributed throughout the spheroids. However, in U-118 MG a marked peak value of [^{3}H]TdR was observed in the peripheral cell layers. A reason for this might have been a rapid intracellular phosphorylation of TdR in intensively proliferating cells. No accumulation corresponding to DNA incorporation could be observed after these short periods. One interesting phenomenon observed was that TTP was found in a more aggregated pattern than TdR (data not shown). This was probably because TTP was restricted to the extracellular spaces, while TdR could easily pass the cell membranes [7].

Glucose (mol. wt. 180) had even more efficient penetration than TdR and TTP into U-118 MG spheroids (Fig. 3). Already after 0.5 min glucose was found isotropically distributed inside the spheroids. A slight accumulation was observed after 2 and 15 min. Sucrose (mol. wt. 342) penetrated into U-118 MG spheroids more slowly (Fig. 3), and a gradient was still visible after 2 min. However, after 15 min sucrose had penetrated completely into

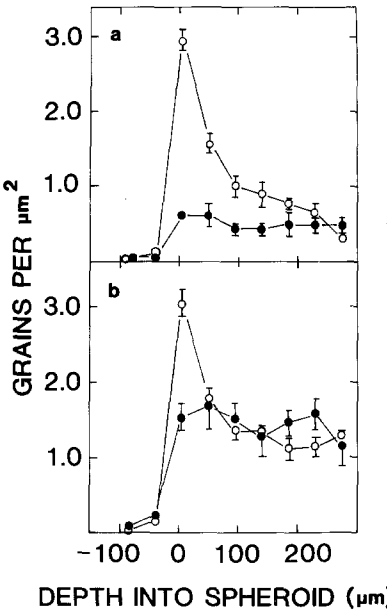

Fig. 1a–b. Concentration of [^{3}H]TdR (○) and [^{3}H]TTP (●), expressed as grain density in contact autoradiograms as a function of depth into U-118 MG spheroids. The penetration patterns after 0.5 min (**a**) and 1.0 min (**b**) are shown. Mean values ± SEM (when exceeding the size of the *symbol*) from six separate evaluations of at least two separate contact autoradiograms are indicated

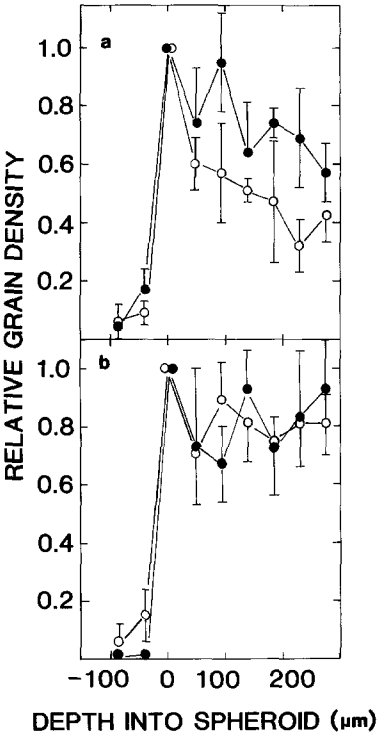

Fig. 2a–b. Concentration of [^{3}H]TdR (○) and [^{3}H]TTP (●), expressed as relative grain density in contact autoradiograms (related to peripheral grain density) as a function of depth into U-393 OS spheroids. The penetration patterns after 0.5 min (**a**) and 1.0 min (**b**) are shown. Mean values ± SEM (when exceeding the size of the *symbol*) from six separate evaluations of at least two separate contact autoradiograms are indicated

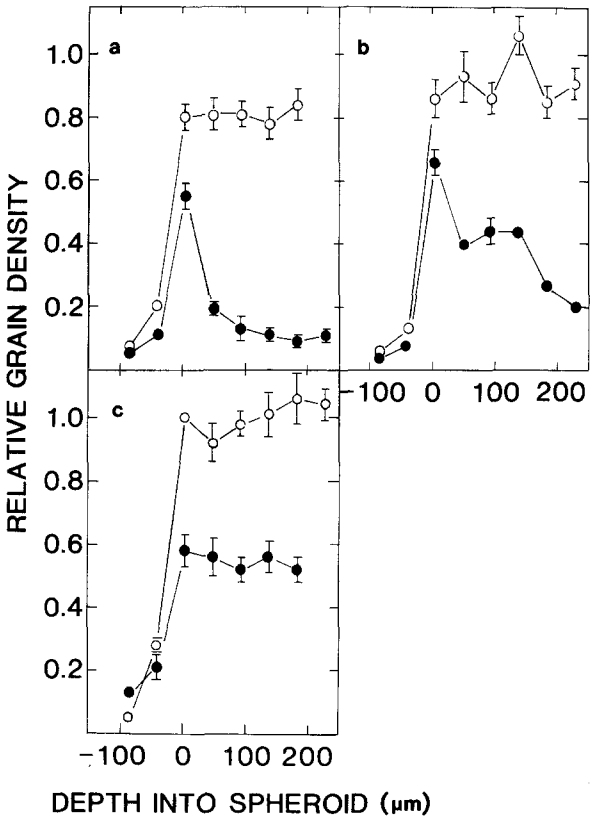

Fig. 3a–c. Concentration of [^{14}C]glucose (○) and [^{3}H]sucrose (●), expressed as relative grain density in contact autoradiograms (related to peripheral density of [^{14}C]glucose after 15 min) as a function of depth into U-118 MG spheroids. The penetration patterns after 0.5 min (**a**), 2 min (**b**) and 15 min (**c**) are shown. Mean values ± SEM (when exceeding the size of the *symbol*) from four separate evaluations of two separate contact autoradiograms are indicated

the spheroids. No differences were observed between the cell lines studied, and some representative contact autoradiograms showing the penetration patterns of glucose and sucrose into HTh-7 spheroids are presented in Fig. 4.

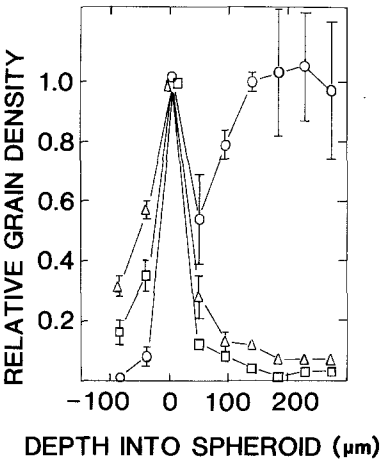

Fig. 5. Concentration of [^{3}H]inulin expressed as relative grain density in contact autoradiograms (related to peripheral grain density) as a function of depth into HTh-7 spheroids. The penetration patterns after 0.5 min (□), 2 min (△) and 15 min (○) are shown. Mean values ± SEM (when exceeding the size of the *symbol*) from four separate evaluations of two separate contact autoradiograms are indicated

Inulin (mol. wt. about 5200) had the slowest penetration of all the substances tested. This substance reached only a few cell layers into HTh-7 spheroids after 0.5 and 2 min; however, after 15 min inulin was found isotropically distributed in the central parts of the spheroids (Fig. 5). In a few cell layers close to the periphery (about 25–100 μm inside) a lower grain density than in the rest of the spheroid was observed even after 15 min (Fig. 5). This might have been due to a high proportion of intracellular space, not allowing the presence of inulin, in this region. The fine structure of inulin distribution inside the spheroids was heterogeneous, probably reflecting the distribution between intracellular and extracellular space. The penetration pattern of inulin was also similar for the cell lines studied, and some representative contact autoradiograms from U-118 MG are shown in Fig. 6.

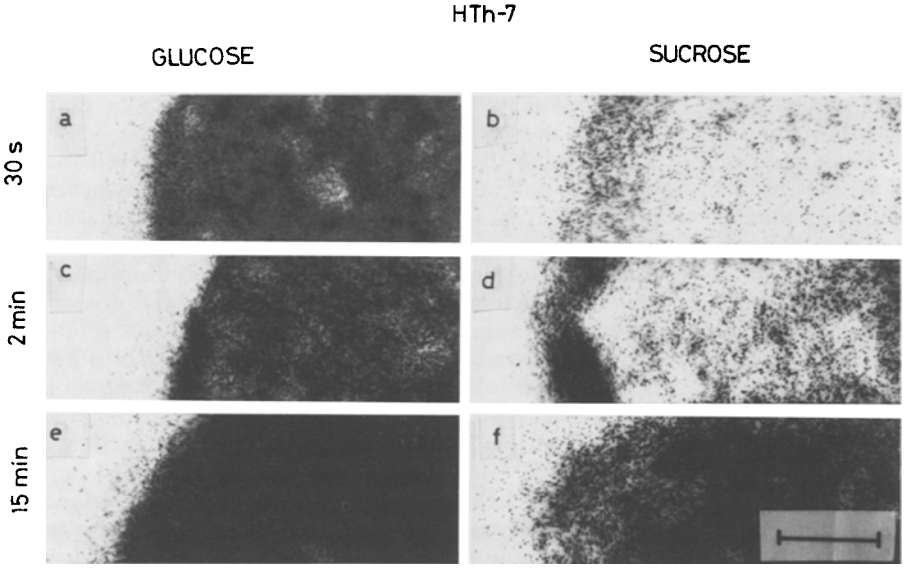

Fig. 4a–f. Contact autoradiograms showing the penetration pattern of [^{14}C]glucose (**a, c, e**) and [^{3}H]sucrose (**b, d, f**) in HTh-7 spheroids after incubation for 0.5 min (**a, b**), 2 min (**c, d**) or 15 min (**e, f**). During the autoradiographic exposure the periphery of a spheroid was located at the edge of the high grain density to the left, while the central parts were to the right in the photographs. *Bar*, 100 μm

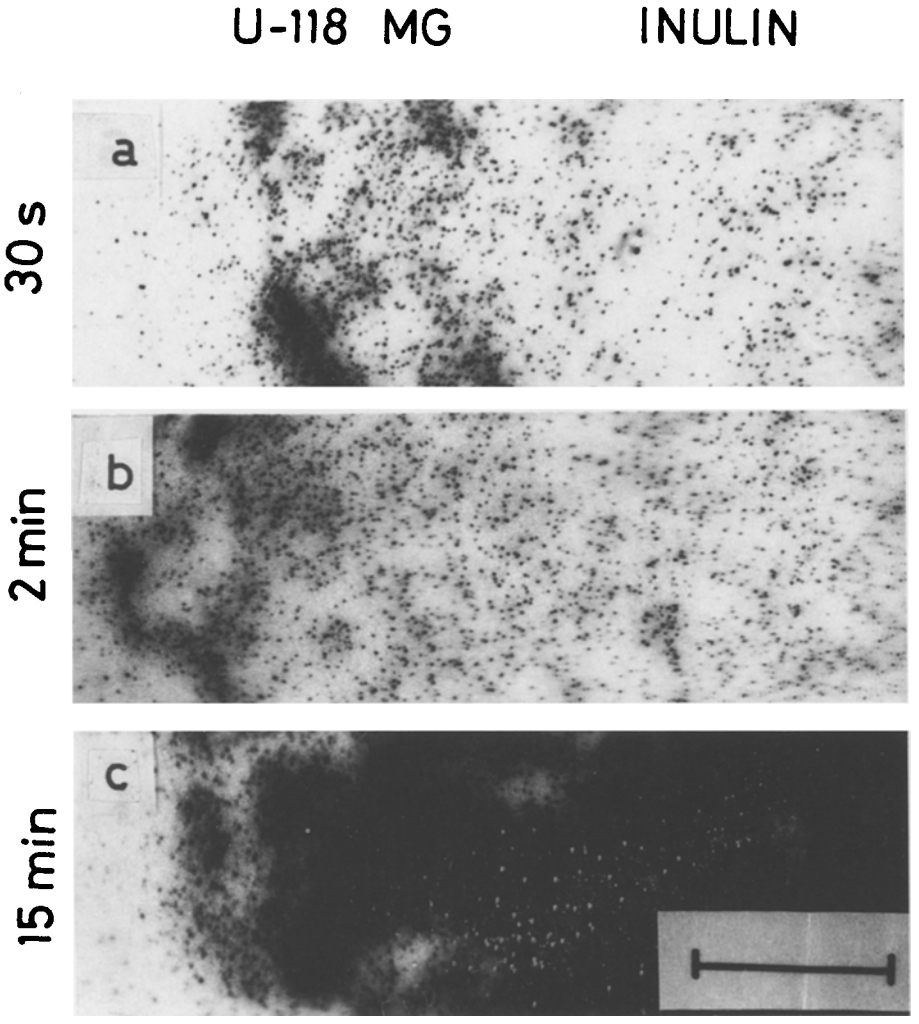

Fig. 6a–d. Contact autoradiograms showing the penetration patterns of [^{3}H]inulin in U-118 MG spheroids after incubation for 0.5 min (**a**), 2 min (**b**) or 15 min (**c**). During the autoradiographic exposure the periphery of a spheroid was located at the edge of the high grain density to the left, while the central parts were to the right in the photographs. *Bar*, 100 μm

Discussion

In order to evaluate the relevance of these results, the properties of the different substances must be known. The nucleoside TdR is known to pass easily through the membranes of mammalian cells [7], while the triphosphorylated derivative TTP, owing to its charged phosphate groups, which give it a strongly hydrophilic nature, is unable to cross the lipid bilayer of membranes. Nothing is known about the diffusion coefficient of these substances in the used cellular and extracellular matrix system. However, assuming a simple aqueous solution, the diffusion time into the central parts of the spheroids, according to the Einstein-Smoluchowski equation [21] $x^2 = 2Dt$ (where x = mean diffusion length in centimetres, D = diffusion coefficient in water in square centimetres per second, and t = time in seconds) should be in the order of 1 min for both these substances.

On the basis of their hydrophilic nature, none of the sugars would be expected to be able to diffuse through cell membranes. However, glucose, although insoluble in lipids, can easily pass through the membranes by facilitated diffusion [11, 25], i.e. with the help of a carrier substance. This process does not exist for sucrose (disaccharide) or inulin (polysaccharide). In fact, these substances, owing to their inability to cross mammalian cell membranes, have been used to measure the extracellular fluid volume of mammalian tissue and cell cultures [9, 10, 23]. Assuming the same conditions as in the previous discussion about TdR and TTP, the diffusion time into the central parts of spheroids should be approximately 1 min for glucose and sucrose, and about 5 min for inulin.

Although the ability to cross mammalian cell membranes is quite different for TdR and TTP, their penetration properties were very similar. This indicated that there was no need for the substances to pass through the cells in order to reach the central parts of the spheroids rapidly. In fact, the penetration of TTP was so rapid that it was in accordance with undisturbed diffusion in aqueous solution.

Also in the case of the sugars, there seemed to be no need for transcellular penetration to reach the central parts of spheroids. Both sucrose and inulin reached the central parts in spite of their inability to cross mammalian cell membranes. However, their penetration seemed to be slightly slower than the calculated theoretical diffusion for globular molecules in aqeuous solutions. Glucose had the fastest penetration, followed by sucrose. Since the degree

of water solubility is similar for glucose and sucrose, the difference in penetration velocity may reflect their different ability to penetrate through the cells. Also, the slight difference in molecular weight may be involved. It might be mentioned that in some experiments [^{14}C]mannitol (mol. wt. 184, unable to pass through cell membranes) was also used as a model substance. This sugar alcolhol seemed to be intermediate between glucose and sucrose in penetration capacity (data not shown), which fits in with the present discussion. The considerably slower penetration observed for inulin may be a result of its higher molecular weight. Inulin is also somewhat less hydrophilic than the other saccharides. However, the capacity of inulin to reach the central parts of spheroids indicates that extracellular penetration is a possible mechanism even for rather large molecules.

To summarize, all the tested substances had a rather free diffusion path into the central parts of the spheroids. The penetration properties of TTP were indistinguishable from those of TdR. The difference between glucose and sucrose indicates the possibility of a difference in rate between free penetration (both transcellular and extracellular) and exclusively extracellular penetration.

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Received May 28, 1987/Accepted January 11, 1988