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# Penetration and binding of vinblastine and 5-fluorouracil in cellular spheroids

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***Summary.*** *The kinetics of the penetration and binding of the two commonly used antitumour drugs vinblastine and 5-fluorouracil in nonvascularized tumour tissue were studied. Multicellular human tumour spheroids (glioma U-118 MG and thyroid cancer HTh-7) were used as a model system. Radiolabelled drugs were used in all studies. To avoid disturbances in the distribution of unbound drugs a dry histological technique was used in combination with contact autoradiography. In addition, quantitative measurements of the accumulation and binding of the drugs were made. The results showed that vinblastine penetrated the spheroids less efficiently than 5-fluorouracil. Vinblastine required about 2 h to be isotropically distributed within the studied spheroids, while only a few minutes were required for 5-fluorouracil. Vinblastine seemed to be accumulated in the peripheral parts of the spheroids within 15 min. High concentrations of 5-fluorouracil, isotropically distributed in the spheroids, were observed after 2 h of incubation. Significant amounts (about half) of the accumulated drugs resisted gentle washing for 3 × 20 s plus 15 min in fresh medium. The limited penetration of vinblastine correlated well with a previously observed high resistance of spheroids to treatments of short duration with this drug.*

## Introduction

Several properties of antitumour drugs are of importance for the therapeutic effects. It is desirable that an antitumour drug show specific toxicity towards tumour cells, and that the drug can reach all the cells in the tumour. One reason for the unsuccessful results of chemotherapy of solid tumours sometimes observed might be insufficient penetration of the drugs into poorly vascularized regions of the tumours. The dose of the drug in the tumour is also dependent on the accumulation in the tissue and the adhesion to cellular structures. In particular, in cases when a drug has a fast clearance from the blood it is of interest to know whether it penetrates efficiently or not and whether it is bound or easily washed out of the tissue again.

Cellular spheroids have been used as an in vitro model system for poorly vascularized nodules of solid tumours both in radiotherapeutic [2, 5, 16] and chemotherapeutic [9] studies. The spheroids seem to offer many of the characteristics of in vivo tumours which are unavailable in monolayer or suspension cultures; for example, necrotic processes, proliferation, and oxygen gradients [1, 16, 18], and the presence of an organized extracellular matrix [14].

The two commonly used antitumour drugs vinblastine [15, 21] and 5-fluorouracil [7, 8] have previously been shown to differ in their ability to penetrate into spheroids [12]. We have now studied the kinetics of the penetration of the two drugs in more detail, and also their ability to resist washout from the spheroids. Special emphasis was laid on the difference between penetration and binding.

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## Material and methods

*Cell culture.* Two human tumour cell lines established at the Wallenberg laboratory (Uppsala, Sweden) were used: the glioma cell line U-118 MG and the thyroid cancer cell line HTh-7. These spheroids were chosen because they are previously well characterized regarding (for example) formation and growth [1], effects of vinblastine and 5-fluorouracil [11], penetration of different substances [10, 12, 13], and extracellular matrix production [14]. It should be noted that these spheroids were not primarily used as model systems for gliomas and thyroid cancers, respectively, but merely as a model system for the poorly vascularized regions of any solid tumor. The cells were cultured as spheroids using the liquid-overlay technique [4, 9, 22]. The culture medium used was Ham's F10 supplemented with 10% newborn bovine serum, L-glutamine (2 m*M*), streptomycin (100 m*M*/ml), and penicillin (100 IU/ml) (Flow Laboratories Swedish AB, Stockholm, Sweden).

*Penetration studies.* The radiolabelled drugs [G-$^3$H]vinblastine sulfate (366 GBq/mmol) and 5-fluorouracil [6-$^3$H]uracil (37 GBq/mol) (The Radiochemical Centre, Amersham, England) were used. To detect both the bound and the soluble fraction of the drugs, a histological method of preserving the water-soluble fraction of the drugs was used. This method has previously been described in detail [12]. Briefly, spheroids attached to round cover slips were incubated in medium containing the radiolabelled drug (1.85 MBq/ml). This concentration of radioactivity was chosen to achieve an appropriate amount of disintegrations in the sections during the autoradiographic exposure. It corresponds to drug concentrations of about 5 μ*M* for vinblastine and 50 μ*M* for 5-fluorouracil. This can be compared with achievable peak plasma levels in man of about 1 μ*M* for vinblastine after IV injection of 0.2 mg/kg [15] and about 700 μ*M* for 5-fluorouracil after IV injection of 10 mg/kg [7]. However, the drug concentrations

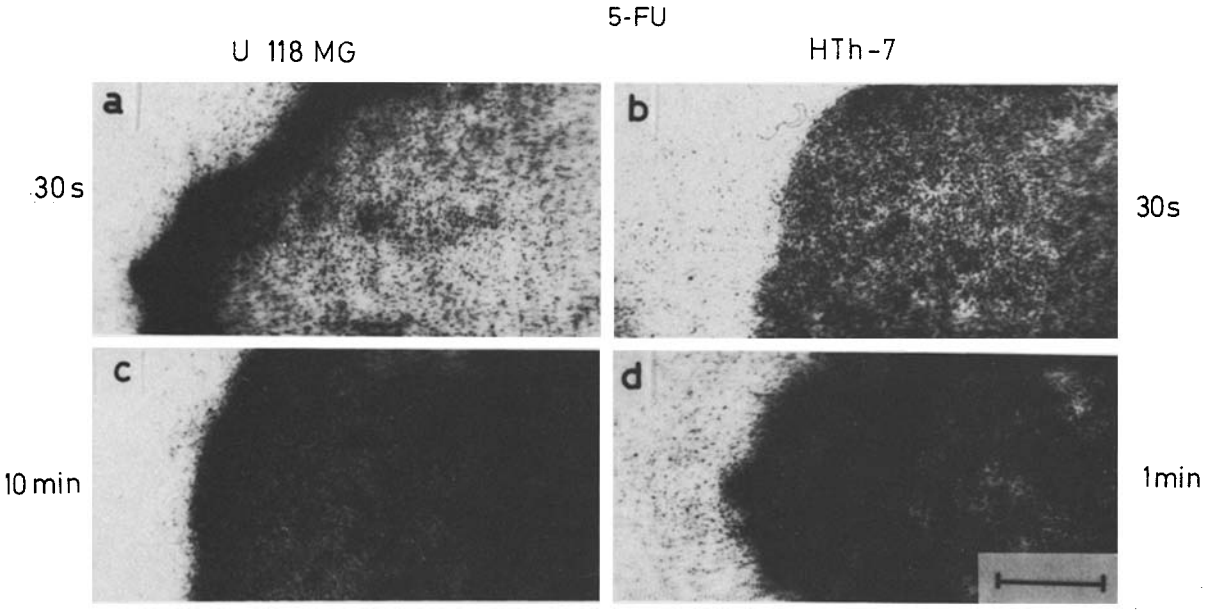


**Fig. 1a–d.** Contact autoradiograms showing the penetration pattern of [$^3$H]5-fluorouracil in glioma spheroids (**a** and **c**) and in thyroid cancer spheroids (**b** and **d**) after incubation for 30 s (**a, b**) 10 min (**c**), or 1 min (**d**). During the autoradiographic exposure the periphery 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. The *bar* indicates 100 μm

cannot be regarded as of great interest in this work, since the spheroids were killed immediately after the drug exposures and the drug effects were not allowed to become biologically manifest. After different incubation times the cover slips were picked up and immersed in a bath of liquid propane-propene mixture kept cool in liquid nitrogen, for fast freezing of the spheroids. The spheroids were then freeze-dried at −80° C to −70° C (TIS-U-DRY, FTS Systems Inc., Stone Ridge, NY, USA), vapour-fixed in a closed chamber containing a paraformic-aldehyde-saturated atmosphere at 80° C, and immersed in xylene to allow proper wax infiltration for the embedding in Histowax (Histo-Lab. Ltd, Gothenburg, Sweden). The embedded spheroids were sectioned, dry-mounted on object plates, and unwaxed in xylene.

Contact autoradiography was used to avoid disturbances in the distribution of the unbound drug also during the autoradiographical procedure. Clean object plates were immersed in photoemulsion (Ilford K5) and allowed to dry in the dark. A plate with dry emulsion and a plate with a spheroid section were put together, so that the section was pressed tightly to the emulsion for 4 weeks to allow autoradiographic exposure. The plate with photoemulsion was then developed in Kodak D19, fixed in Kodafix, and photographed on Kodak Panatomic-X with a microscope camera (Leitz Vario-Orthomat camera and Leitz Orthoplan microscope).

The use of these histological and autoradiographical methods has been shown to prevent any significant disturbances in the distribution of unbound test substances [12]. As a comparison conventional histology and autoradiography, as described by Haji-Karim and Carlsson [4], were also performed.

*Studies of drug-binding properties.* These studies were made qualitatively by the histological and autoradiographical methods described above, and also by quantitative measurements using liquid scintillation counting. Spheroids were then

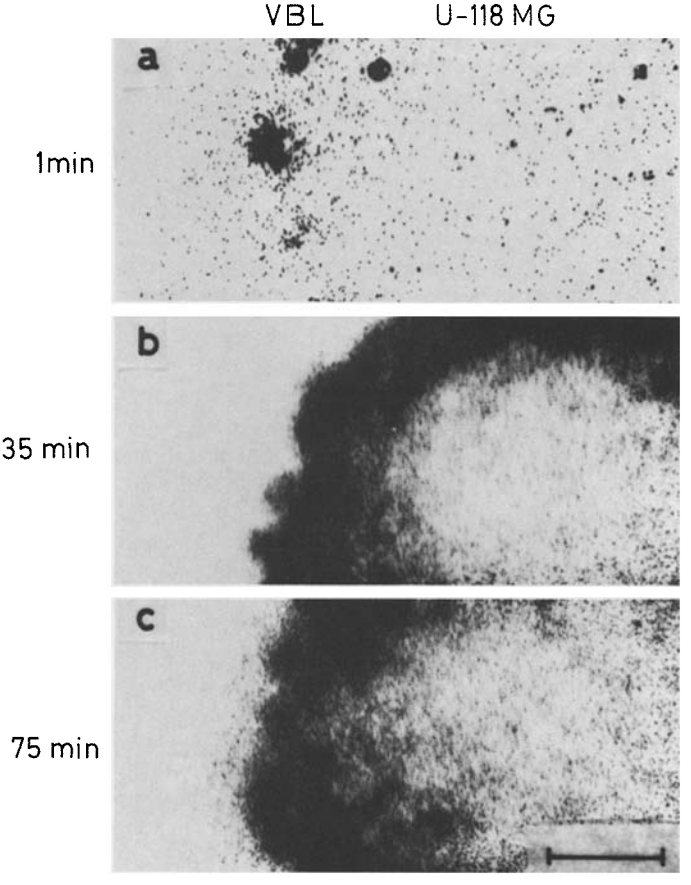


**Fig. 2a–c.** Contact autoradiograms showing the penetration pattern of [$^3$H]vinblastine in glioma spheroids after incubation for 1 min (**a**), 35 min (**b**), or 75 min (**c**). During the autoradiographic exposure the periphery of a spheroid was located at the edge of the high grain density to the *left*, while the central parts were to the *right* in the photographs. The *bar* indicates 100 μm

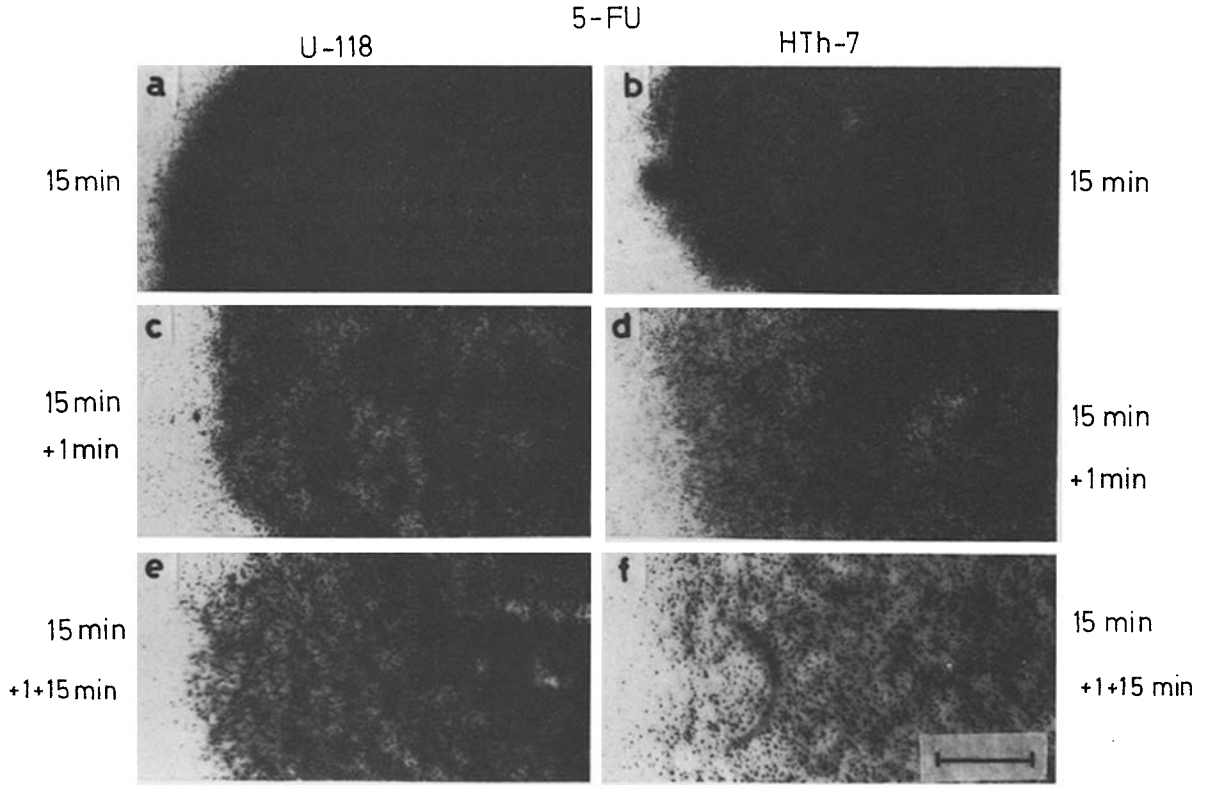


**Fig. 3a–f.** Contact autoradiograms showing the distribution of [$^3$H]5-fluorouracil in glioma spheroids (**a, c,** and **e**) and in thyroid cancer spheroids (**b, d,** and **f**) after 15 min of incubation (**a** and **b**) or 15 min of incubation followed by washing in fresh medium for 1 min (**c** and **d**) or 1 + 15 min (**e** and **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. The *bar* indicates 100 μm

incubated for different times in medium containing the radiolabelled drug. Some spheroids were very quickly washed (about 5 s) in fresh medium after the incubation, just to remove the incubation medium, while others were more carefully washed (3 × 20 s + 15 min) to remove all soluble drug. The spheroids were then dissolved in 1 ml NaOH (0.3 *M* for 24 h). The solutions were neutralized and the content of radioactivity was measured by liquid scintillation counting. The diameter of the spheroids used was 490 ± 86 μm and 724 ± 177 μm (mean values ± SD) for U-118 MG and HTh-7, respectively. No systematic relationship could be observed between the drug-binding properties and the spheroid sizes at the time of drug exposures.

## Results

### *Drug penetration*

In accordance with previous results [12], a marked difference was demonstrated between the two drugs (Figs. 1 and 2). 5-Fluorouracil seemed to have penetrated efficiently within a few minutes. The only case where a slight gradient could be observed was in glioma spheroids after 30 s of incubation. Vinblastine, however, had a limited penetration. After 1 min of incubation hardly any drug had penetrated into the glioma spheroids and a gradient persisted even after as long an incubation time as 75 min.

### *Drug washout*

No gradient in drug concentration seemed to persist in the spheroids after 15 min of incubation in medium with 5-fluorouracil or 2 h of incubation in medium with vinblastine. After these incubation periods spheroids were washed for different times in fresh medium and then analysed by contact autoradiography. The autoradiograms from these experiments are shown in Figs. 3 and 4. Significant amounts of the drugs could still be detected in the spheroids after washings in fresh medium for 1 plus 15 min. However, the drugs in the spheroids did not resist conventional histological procedures with methacrylate embedding. After 2 h of incubation in either of the drugs no grains could be detected over the spheroid sections when conventional techniques, including fixation in methanol : acetic acid (3 : 1), dehydration in ethanol, infiltration by methacrylate and dipping autoradiography, were used.

### *Drug accumulation and binding*

Liquid scintillation counting of spheroids incubated for 15 min or 2 h in medium containing either of the drugs and only quickly washed (5 s) in fresh medium showed that both the drugs could be significantly accumulated in the spheroid tissue compared with the incubation medium (Table 1). The accumulation was higher for vinblastine than for 5-fluorouracil, and in most cases it also seemed to be higher in the glioma

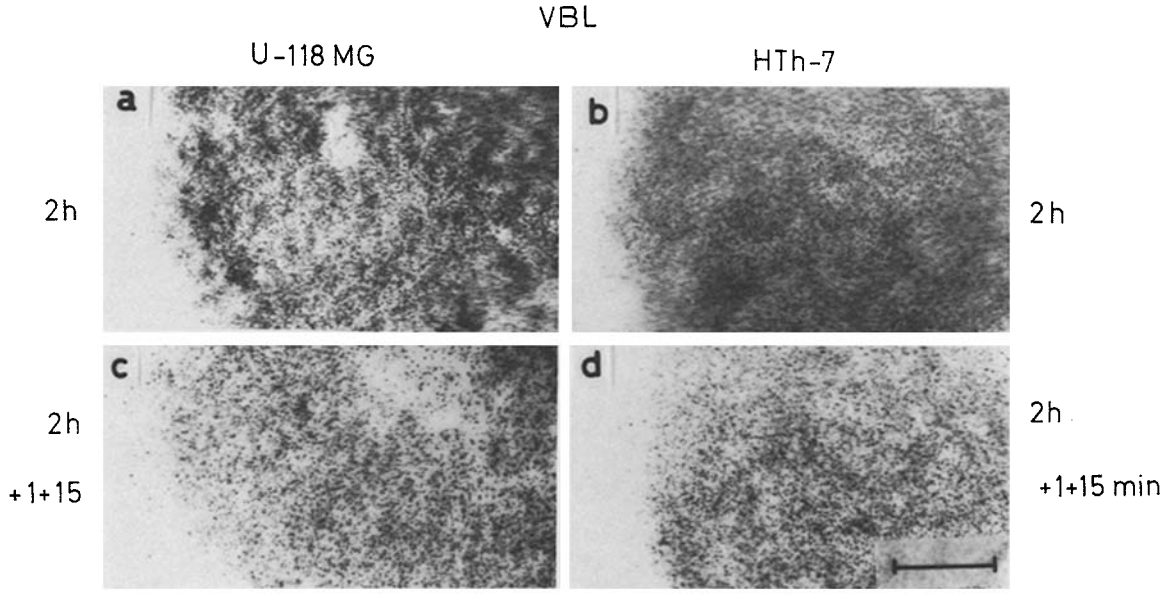


**Fig. 4a–d.** Contact autoradiograms showing the distribution of [$^3$H]vinblastine in glioma spheroids (**a** and **c**) and in thyroid cancer spheroids (**b** and **d**) after 2 h of incubation (**a** and **b**) or 2 h of incubation followed by washing in fresh medium for 1 + 15 min (**c** and **d**). 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. The *bar* indicates 100 μm

spheroids than in the thyroid cancer spheroids. In all cases, about half (40%–70%) the accumulated drug still remained in the spheroids after washing for 3 × 20 s plus 15 min in fresh medium (Table 2).

**Table 1.** Concentration of radiolabelled drug in spheroids in relation to the incubation medium[a]

| Drug[b] | Spheroid type | Relative radioactivity after | |
|---|---|---|---|
| | | 15 min | 2 h |
| VBL | U-118 MG | 6.7 ± 1.2 | 8.2 ± 1.6 |
| VBL | HTh-7 | 3.5 ± 0.2 | 9.7 ± 0.3 |
| 5-FU | U-118 MG | 1.4 ± 0.1 | 4.9 ± 0.6 |
| 5-FU | HTh-7 | 0.8 ± 0.1 | 2.2 ± 0.3 |

[a] Concentration of radioactivity in the incubation medium was set at 1. After the incubations the spheroids were quickly immersed (about 5 s) in fresh medium just to remove the incubation medium. The values are mean values ± SD from measurements of at least three individual spheroids. Spheroid sizes are given in the text

[b] VBL, [$^3$H]vinblastine; 5-FU, [$^3$H]5-fluorouracil

**Table 2.** Remaining drug after washing for 3 × 20 s + 15 min in fresh medium of spheroids incubated for 15 min or 2 h in medium containing radiolabelled drug[a]

| Drug[b] | Spheroid type | 15 min | 2 h |
|---|---|---|---|
| VBL | U-118 MG | 49 ± 3 | 45 ± 16 |
| VBL | HTh-7 | 56 ± 6 | 41 ± 4 |
| 5-FU | U-118 MG | 40 ± 2 | 63 ± 11 |
| 5-FU | HTh-7 | 59 ± 2 | 68 ± 14 |

[a] Percentages of the originally accumulated radioactivity (see Table 1) are given. The values are mean values ± SD from measurements of at least three individual spheroids

[b] VBL, [$^3$H]vinblastine; 5-FU, [$^3$H]5-fluorouracil

## Discussion

Some studies of drug penetration into spheroids have previously been performed using standard histology [3, 17, 19]. It is, however, important to remember that probably only covalently bound drugs can be detected by such methods, while loosely attached or unbound drugs will be washed away during the histological procedures. The 'dry' histological and autoradiographical methods used in this work have been shown also to preserve the unbound fraction of the drugs [12]. No radioactivity could be detected in spheroid sections after conventional histology.

Higher resistance of spheroid cells than of monolayer cells has previously been demonstrated for vincristine [20]. A similar effect has recently been shown also for vinblastine [11]. However, in the latter study it was also demonstrated that spheroids had an extra resistance to treatments of short duration (15 min) with vinblastine. This extra resistance could not be demonstrated for 5-fluorouracil treatments or long-term vinblastine treatments. These data correlate well with the penetration patterns shown in this study.

The limited penetration of vinblastine indicates that there might be suboptimal conditions for chemotherapy of poorly vascularized tumours with this drug, especially since the half-life of vinblastine in the serum is very short (about 5 min) in the first phase after IV injection [15]. However, liquid scintillation counting showed that both the drugs were accumulated in the spheroids. A similar effect has previously been demonstrated for HeLa cells exposed to vinblastine [6], indicating that the drug concentration in some parts of a tumour might be higher than in the serum. Furthermore, the results from the drug-binding studies indicated that the clearance from unvascularized tissue might be slower than the clearance from the blood.

It can be concluded that not only toxicity but also penetration and binding should be taken into account to some degree when in vitro studies of cytotoxic drugs are performed.

Better knowledge of these factors is of importance for the development of new drugs.

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