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In-beam light absorption measurement of anthracyclines in cells with a flow-through system

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A flow-through cuvette in which cells attach as a monolayer to a quartz plate was developed for measurement of the light absorbance of anthracyclines in cells. Despite the drawback of a short path-length (of the order of the cell diameter), a dynamic flow-through set-up and baseline storage made it possible to measure intracellular absorbance and obtain spectral data for daunomycin and carminomycin. Stopping the flow and allowing the drug to equilibrate between medium and cells led to a 20% decrease of molar light absorption of cellular anthracycline, which permitted measurement of the total cellular concentration. Furthermore, accumulation and efflux kinetics were determined for H35 rat hepatoma cells. On the basis of the reported formation constant of the iron-complex of carminomycin, which is of the order of 10^{34}, we expected to find this complex within the cells. However, the spectrum of cellular drug did not show absorbance bands characteristic of the complex. A red shift and hypochromism were found in the daunomycin spectrum after intracellular binding, which corresponds with the spectral change observed after intercalation of daunomycin into DNA.

Introduction

Anthracyclines are widely used against a variety of malignant diseases [1]. A correlation between the degree of cellular accumulation and the polarity of various anthracycline analogs has been reported [2]. Strong intracellular accumulation of, e.g., the most apolar analogs, can lead to concentrations up to several hundred times higher than those in the surrounding medium. Daunomycin and carminomycin are among the most apolar analogs. Fluorescence data for anthracyclines in cells have been obtained by flow cytometry [3,4] and digitized video monitoring [5]. Even fluorescence emission spectra have been recorded for cytoplasmic and nucleus-bound drug [6]. Compared with the free drug, intercalation of daunomycin in calf thymus DNA led to a much greater decrease of fluorescence (up to 5% of the signal) than of absorption (up to 65% of the absorbance at 480 nm), and was accompanied by a red shift of 25 nm [7]. This means that for DNA-intercalating anthracyclines, light absorption is less dependent on intracellular compartmentalization and is therefore preferable to fluorescence measurement for direct (semi-) quantitative analysis of the amount of drug taken up by the cell. For anthracyclines, spectral absorbance data obtained in cells can also provide information on the intracellular presence of drug-iron complexes, which show a characteristic absorption

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band in the 550–650 nm wavelength range [8] and have very high formation constants [9,10].

In monolayer cells, however, losses due to light scattering and the limited optical pathway reduce the usefulness of light absorption measurements. Measurement of changes in absorption in a dynamic way can improve the detectability of intracellular drug, because variations in light absorption due to cuvette changing and mixing are avoided. Changes in light absorption by the cells can be minimized by keeping the cellular environmental conditions constant, and this can be achieved by perfusion. The construction of the flow-through perfusion system has been described in an earlier report covering interactions between anthracyclines and cells and dealing with measured changes in the composition of the perfusate [11].

The present paper describes a procedure for obtaining spectral data by the direct measurement of intracellular absorption by drugs with high levels of cellular accumulation. This allows comparison of spectral data both before and after drug exposure during perfusion.

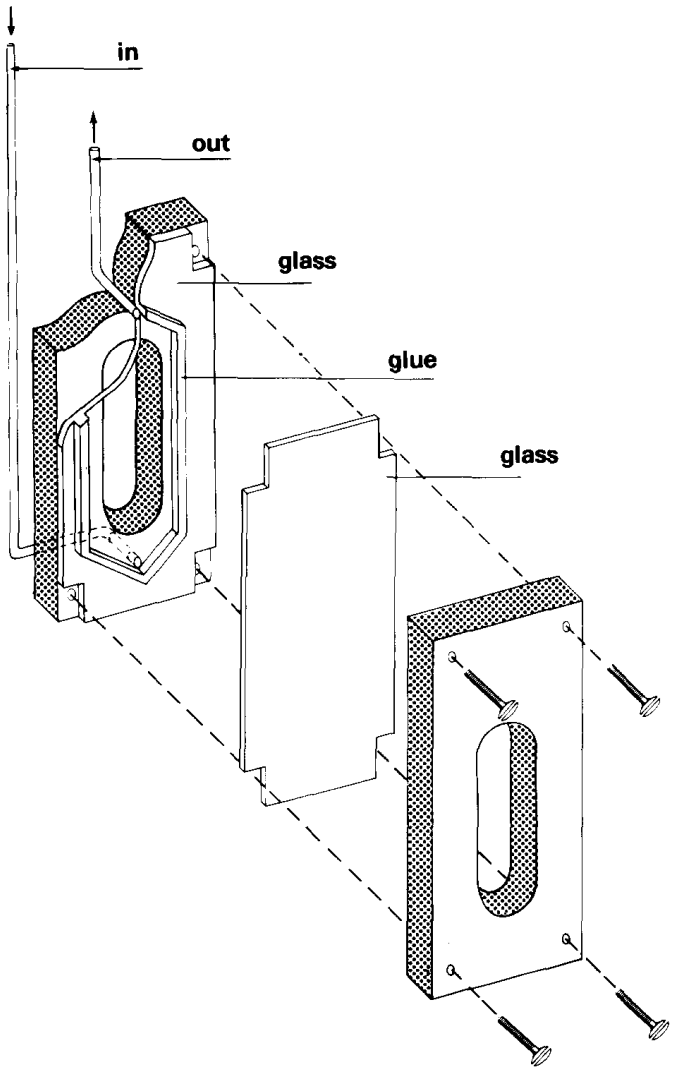

Fig. 1. Schematic representation of the flow-through cuvette. The two deck plates are pressed together by tightening the screws, which forms the cell cavity. Outer dimensions: 1×1 cm. The thickness of the silicone glue layer was between 0.08 and 1 mm. At wavelengths higher than 400 nm, either quartz or glass plates can be used.

Materials and Methods

Cells

H35 rat hepatoma cells [12] were cultured in Dulbecco's modified minimum essential medium (Grand Island Biological Co., Paisley, U.K.) supplemented with 10% fetal calf serum. Poly-(L-lysine) (Sigma) in Hanks' buffered salt solution (1 mg/ml) was used to pretreat the quartz plates for 15 min, after which the plates were rinsed with phosphate-buffered saline (pH 7.4). H35 cells in a trypsinized suspension were allowed to attach to the quartz plate at 37 °C. Glass plates could be used as well, allowing attachment of cells without poly-(L-lysine) pretreatment. Mostly the cells had formed a confluent layer after 1 day. Then the plate was mounted in the perfusion chamber after removal of cells upstream from the light path. Cells were perfused with phosphate-buffered saline (Dulbecco) supplemented with vitamins for minimal essential medium according to Eagle (modified) (Flow Laboratories, Irvine, U.K.) and glucose (10 mM) at pH 7.4. The average cell diameter was calculated from measurements made with an Elzone® monitor (type 80XY, Particle Data Inc., Elmhurst, IL, U.S.A.).

Perfusion chamber

A schematic representation of the perfusion chamber is shown in Fig. 1. When the quartz plates (1 mm thick) are pressed together, a cavity for the cells is formed by a silicone glue spacer (thickness 0.08–1 mm). The lower connecting tube is used as the inlet tube. The cuvette is placed in a scanning spectrophotometer with a bandwidth of 4 nm (Kontron, type Uvikon 722 LC) and a spectral baseline memory option. The cuvette holder is thermostated at 37 °C by air heating and a regulatory system with a thermocouple, the reference temperature being that of the water in the waterbath. The stock of perfusion medium is stirred at 37 °C to prevent degassing during heating, and is pumped with an HPLC pump equipped for micro liquid chromatography (Gilson, type 302). The connecting tubes are made of stainless steel and fused silica. Plastic tubing was excluded to avoid gas exchange. Upstream from the perfusion chamber, an HPLC injection valve with a loop with a volume of 13.5 µl was used for pulse injection of drug into the flowing perfusion medium. For stepwise changes the perfusion medium was divided into two portions, one of them being supplemented with the drug. During stepwise changing of the medium, the medium was flushed through rapidly.

Chemicals

Daunomycin was kindly provided by Specia (Paris, France) and carminomycin by Bristol Myers (Syracuse, NY, U.S.A.). Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) was purchased from Janssen Chimica (2340 Beerse, Belgium). Dulbecco's phosphate-buffered saline was obtained from the Grand Island Biological Co. All chemicals were of analytical grade. Ammonium iron(II) sulphate was purchased from Merck (Darmstadt, F.R.G.).

Results and Discussion

Baseline stability

After the quartz plates with the attached cells had been installed in the perfusion chamber, the cells were allowed to equilibrate for 10 min at a perfusion flow rate of 100 µl/min. The spectral baseline was then recorded for wavelengths between 400 and 900 nm and stored in the memory of the spectrophotometer. The uncompensated spectrum of the cells is shown in Fig. 2A. The compensated spectrum was recorded for various periods during perfusion at a flow rate of 100 µl/min (Fig. 2B). The stability of the spectrophotometer was 0.0015 absorbance unit/h between 700 and 900 nm and $4 \cdot 10^{-4}$ absorbance unit/h between 700 and 400 nm. This means that changes in the absorption by the cells were responsible for the variations illustrated in fig. 2A and B.

Light absorption of cellular drug

For various concentrations of daunomycin and carminomycin in phosphate-buffered saline up to 10^{-3} M in medium and in the absence of cells, the absorption was measured in the flow-through cuvette using a spacer thickness of 1 mm. At 480 nm the absorption was linear with the concentration. This makes the method preferable to fluorescence measurement, which lacks linearity above 10 µM (as measured by us for daunomycin). On this

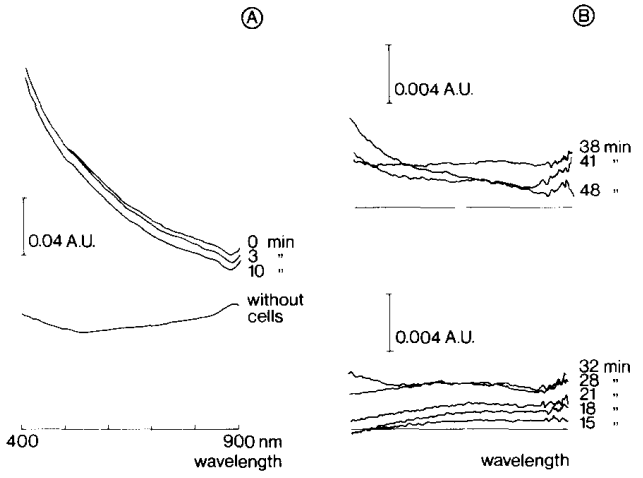

Fig. 2. Absorption spectra of cells. (A) Baseline absorption of H35 cells at various intervals after installation of cells. (B) Baseline absorption after subtraction of the baseline determined after the first 10-min interval. Note that the scale is 10-times more expanded than in (A). A.U., absorbance unit.

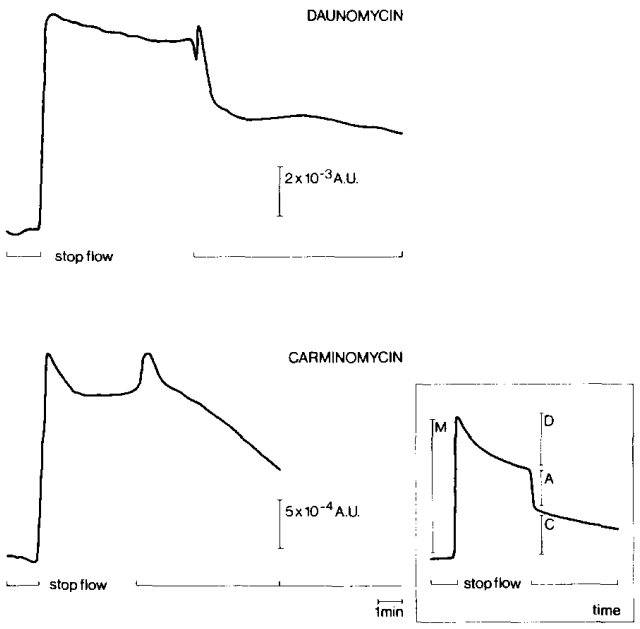

Fig. 3. Measurement of light absorption during accumulation of drug by cells ($\lambda = 480$ nm). During the accumulation phase, the flow of the medium was stopped and absorption decreased due to uptake of the drug by the cells (spacer thickness: 80 μm). Inset: Standard curve used to calculate the cellular absorbance efficiency factor (k). Drug concentrations in the medium at the start of drug accumulation were 24 μM (carminomycin) and 100 μM (daunomycin). A.U., absorbance unit.

basis, a decrease in the apparent molar absorption coefficient of the drug due to local cellular concentration effects was not to be expected for light absorption measurements in the presence of cells. For monitoring of the change in molar absorption occurring when the anthracycline moves from the medium into cells, we used a thin spacer of 0.1 mm to obtain a minimal diffusion distance. After injection of a pulse of daunomycin or carminomycin, the absorption at 480 nm increased and the flow was stopped immediately after maximum absorption was reached. The subsequent accumulation of the drug in the cells led to a slight decline of the total absorption, as shown in Fig. 3. When the flow was restarted, the medium was rapidly cleared of drug and then the slower phase of efflux from the cells was observed.

When the cellular molar absorption is k-times the molar absorption of the drug in medium, the following equation can be derived. If D is the absorbance reduction (Fig. 3) due to intracellular accumulation and C is the absorption of intracellular drug, the amount transferred to the cells is represented by $M - A$, which equals $D + C$. Since absorption by the cellular drug is C, we have $k = C/D + C$.

This factor was calculated for daunomycin and carminomycin under the conditions shown in Fig. 3, and proved to be 0.81 for both daunomycin and carminomycin. Resumption of medium flow led to a slight peak (Fig. 3) appearing as a continuation of the original peak and representing anthracycline upstream from the cells. For carminomycin this second peak is relatively broad and might be responsible for an erroneous value of A suggesting extra accumulation during this peak. However, because almost all of the dose of carminomycin is now cellular carminomycin, A can be neglected in this case and M can be substituted for $D + C$. For daunomycin a similar decrease in absorption was observed by Chaires et al. [7] upon binding to DNA. For daunomycin and carminomycin (50 μM in phosphate-buffered saline) we found fractions amounting to 0.8 and 0.7 of the absorption at 480 nm, respectively, after addition of DNA in excess (0.25 mM). Comparison of these authors' data with our findings suggests intercalation of daunomycin and carminomycin into DNA. In

contrast, the decrease in fluorescence during DNA binding reported by the same authors was much greater. The wider linearity range and the smaller decrease in optical sensitivity are advantages offered by light absorption measurement over fluorescence measurement for the quantitative estimation of cellular drug. This procedure can be used to study the dynamics of cellular uptake and efflux, but cannot equal the lower detection limit of fluorescence signals and the information that can be obtained from fluorescence anisotropy and fluorescence lifetime measurements.

The two methods could, however, be used complementarily, e.g., in the case of high quenching.

Cellular accumulation

Fig. 4 shows the light absorbance recorded at 480 nm when the medium without drug was supplemented stepwise with daunomycin or carminomycin at concentrations of 25 μM and 10 μM, respectively. During the 'accumulation phase', measured at continuous flow conditions, combined uptake and efflux tended to give a steady-state concentration. Switching to drug-free medium led to a sudden drop of the drug concentration in the medium, followed by an 'efflux phase'.

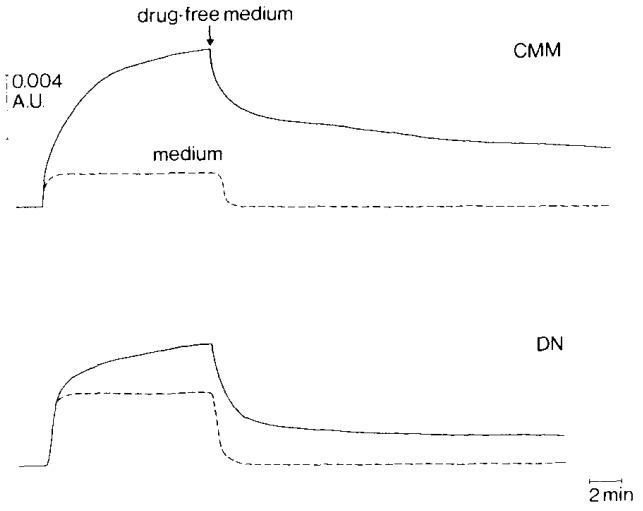

Fig. 4. Ingestion of carminomycin (CMM; 10 μM) and daunomycin (DN; 25 μM) by H35 cells; after the period of accumulation, the cells were given drug-free medium ($\lambda = 480$ nm). The dashed line represents the light absorption by the drug in the medium (liquid film thickness: 0.16 mm). A.U., absorbance unit.

Calculated on the basis of this decline, the efflux half-life time was about 10 and 18 min for daunomycin and carminomycin, respectively. On the assumption of an average cell path-length of 2 μm (calculated from the average cell diameter and surface coverage) and with use of the calculated k value (see above), the average cellular concentration was estimated to be $1.4 \cdot 10^{-3}$ M and $3 \cdot 10^{-3}$ M for daunomycin and carminomycin, respectively, which is roughly 55- and 300-times the concentration of daunomycin and carminomycin in the medium. For daunomycin the cellular accumulation factor in L1210 cells measured after the same incubation time with $3 \cdot 10^{-6}$ M drug [2] was about 100. This compared well with our finding. At 6 pg DNA per cell, the mean ratio of nucleotide to drug molecules was 10:1 for daunomycin and 5:1 for carminomycin.

Absorption spectra of cellular daunomycin and carminomycin

Spectra were obtained for daunomycin and carminomycin in medium with and without cells after the injection of a pulse of the drug followed by accumulation for 5 min and flushing away of the drug-containing medium (see Fig. 5). Immediately before addition of the drug the background spectrum was recorded for subtraction from the other spectra. During scanning, the flow rate of the medium was reduced to 5 μl/min to reduce the chance of changes in concentration during perfusion. The graphs have been adjusted to permit comparison of the spectra. As can be seen from Fig. 5, the peak maxima of both compounds shifted to higher wavelengths during cellular accumulation. These observations, in combination with the decrease of the molar absorption, correspond with the spectral effects (hypochromism, red shift) observed for daunomycin after binding to DNA [7].

Spectra of the carminomycin-iron complex

After titration of carminomycin with a solution of ammonium iron(II) sulphate, the spectrum of the free drug changed to that of the iron complex, with a stoichiometric drug-to-iron ratio of 2:1, as previously reported [8] and shown for carminomycin in Fig. 6, where a characteristic absorption band at 550–650 nm can be seen. When the

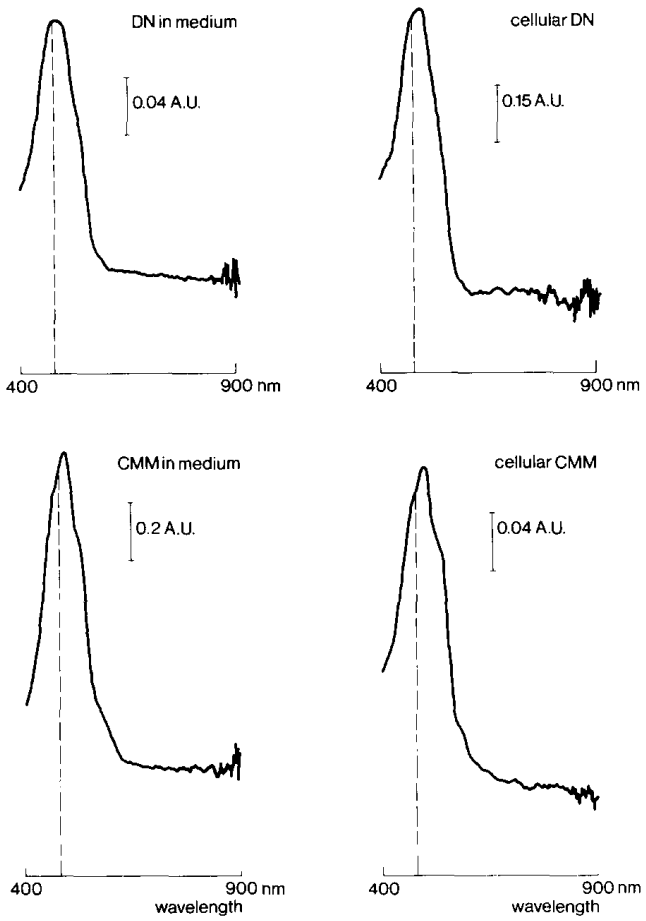

Fig. 5. Daunomycin (DN) and carminomycin (CMM) spectra recorded after addition of the drug to the medium without cells, and after 5 min of accumulation in H35 cells followed by removal of the medium containing the drug. Scanning rate: 200 nm/min; 480 nm is indicated by the dashed line. A.U., absorbance unit.

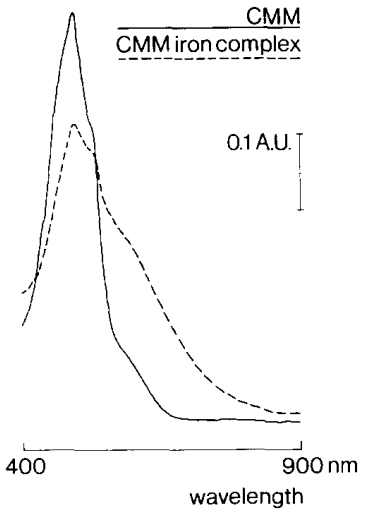

Fig. 6. Visible absorbance spectrum of 47 μM carminomycin (CMM)-iron complex (------) and 47 μM free carminomycin (———). Light path-length: 1 cm at a molar drug/iron ratio of 2:1 in 0.05 M Hepes buffer (pH 7.2). A.U., absorbance unit.

concentration was just under the equivalence point (10% excess of carminomycin), the iron complex was stable in the perfusion medium. Having assumed an average total cellular iron concentration of the same order of magnitude as that reported for CHO cells, i.e., 1 mM [13], or for heart tissue, i.e., 0.1 mM [14], we had expected to find the complex in an intracellular localization immediately after the uptake of carminomycin and daunomycin, in view of the very high formation constant of the order of 10^{34} for carminomycin [10] and 10^{28} for daunomycin [9].

This absence might have been due to the formation of an intermediate iron-carminomycin complex followed by binding of iron to phosphate groups of DNA, as suggested by Beraldo et al. [9]. Destabilization of the iron-daunomycin complex did occur, but appeared to be a slow process, of the order of hours, as reported for daunomycin by the same authors [9]. To see whether this also holds for carminomycin, we used phosphate-buffered saline (pH 7.4) as solvent and incubated the carminomycin-iron complex (10^{-5} M) with calf thymus DNA ($5 \cdot 10^{-4}$ M nucleotides); the results showed that within 2 h the spectrum changed to that of DNA-bound carminomycin. Another possible explanation of the absence of iron is compartmentalization of the metal. Most of the intracellular iron is stored as a ferritin complex. Thomas and Aust have reported an anaerobic release of iron from ferritin that was catalysed by NADPH–cytochrome-*P*-450 reductase [14]. The presence of oxygen, which would keep iron in the oxidized state (in which it cannot easily be removed from ferritin) and thus prevent it from coming into contact with the drug, might also explain the absence of the iron complex. Another attempt to find out whether the iron complex could be detected in cells was made by injecting the complex into the flowing medium. A detectable amount of the complex did not accumulate in the cells and we observed only a low-intensity

spectrum of cellular carminomycin, which was probably attributable to the small excess of carminomycin added to the iron-complex preparation.

Conclusion

The measurement of absorption proved to be a reliable method for the determination of cellular drug concentrations. The direct measurement of cellular anthracycline absorption spectra is a useful method for determination of the nature of the complex that anthracyclines may form in cells. In view of the high formation constant, we expected that the iron complex would be found to play at least a partial role in the release of drug from cells. However, the iron complex was not detected. The occurrence of cellular resistance to anthracyclines is often associated with reduced cellular drug retention [15]. Changes in the mechanisms underlying retention are of interest in relation to the mechanisms of drug resistance.

In an ongoing study the findings concerning changes in light absorption by anthracyclines will be compared with the results of total cellular anthracycline fluorescence quenching measurements made with the stop-flow technique described in this paper.

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