Continuous in Situ Electrochemical Monitoring of Doxorubicin Efflux from Sensitive and Drug-Resistant Cancer Cells

Chen Yi* and Miklós Gratzl#

*#Department of Biomedical Engineering and #Department of Physiology and Biophysics, Case Western Reserve University, Cleveland, Ohio 44106 USA

ABSTRACT One of the least well understood problems in cancer chemotherapy is the cross-resistance of certain tumor cells to a series of chemically unrelated drugs. Multidrug resistance (MDR) can be attributed to several different biophysical processes, among them increased drug efflux. This has been found to correlate with overexpression of the cell surface 170-kDa P-glycoprotein that actively excludes cytotoxic drugs against their concentration gradient. To better understand MDR, experimental methods are needed to study drug efflux from cancer cells. Continuous measurement of efflux of nonfluorescent drugs on the same cell culture in situ, or assessing efflux from a few cells or even a single cell, is beyond the capabilities of existing technologies. In this work, a carbon fiber (CF) microelectrode is used to monitor efflux of doxorubicin from a monolayer of two cell lines: an auxotrophic mutant of Chinese hamster ovary cells, AUXB1, and its MDR subline, CH^RC5. Because doxorubicin is both fluorescent and electroactive, the results could be validated against existing data obtained optically and with other techniques on the same cell lines, with good agreement found. The electrochemical detection, however, is capable of in situ monitoring with high temporal resolution and is suitable for single-cell studies.

INTRODUCTION

One of the least well understood problems in cancer chemotherapy is the eventual resistance of tumor cells to different chemotherapeutic drugs of natural product origin, such as doxorubicin, actinomycin D, vinblastine, vincristine, or colchinine. Increasing the concentrations of these agents in small consecutive steps results in high-level cross-resistance in such cells to them as well as to many other, chemically unrelated drugs which, nevertheless, tend to be hydrophobic and positively charged (i.e., weak bases) at physiological pH. This phenomenon of multidrug resistance (MDR) can be attributed to several different biophysical processes: decrease in drug uptake, increase in efflux, increase in drug metabolism rate, or alterations in drug-target properties (Michelson and Slate, 1994).

Increased efflux (Inaba et al., 1979; Bradley et al., 1988; Michelson and Slate, 1994) has been found to correlate with the overexpression of the cell surface 170-kDa P-glycoprotein (Pgp), which utilizes the energy of ATP hydrolysis to actively exclude cytotoxic drugs against their concentration gradient, thus increasing efflux, whereas uptake is mainly due to passive diffusion via the plasma membrane (Gottesman et al., 1995). To better understand this important mechanism of MDR, experimental methods are needed to study the dynamic characteristics of drug efflux from sensitive as well as drug-resistant cancer cells.

Three approaches have been used to measure drug efflux: methods involving drug extraction (Astier et al., 1988; Daoud and Juliano, 1989; Marquardt and Center, 1992; Vichi and Tritton, 1992), flow cytometry (Krishan and Ganapathi, 1980; Nooter et al., 1990; Krishan, 1990), and flow-through detection in the culture medium (Spoelstra et al., 1991). Even though all three approaches have been successfully used for efflux studies on cell populations, each has its drawbacks as well. The first one requires complicated, lengthy procedures including incubation, centrifugation, and drug extraction, followed by either different chromatographies or fluorescent and radioactive methods for detection. The cells are destroyed during the procedure, so only one efflux data point for a given efflux period can be obtained from each cell population. The second approach is well suited for quantifying the efflux of virtually any fluorescent drug in cell population studies. It provides high temporal resolution, on the order of one sample per second. However, flow cytometry becomes useless when the studied drug is nonfluorescent or binding to DNA and other target molecules quenches its fluorescence. Moreover, intracelluar pH can affect drug fluorescence, leading to erroneous data (Krishan, 1990). In this methodology, because the cells flow, suspended cells are required. In the last technique, a monolayer of cells is attached to the bottom of a chamber and interacts with the drug-containing medium flowing over the cells. Fluorescence or absorbance of the studied drug in the medium obtained at the outlet of the system is measured and compared to the one in the inlet medium. The change in signal is related to drug uptake or efflux. This technique is designed for cell population studies, and an optical detection scheme is required in the flow-through system. A fluorescent drug is required when low drug concentrations are encountered.

Based on the reported experimental approaches, continuous measurement of efflux of nonfluorescent drugs on the same cell culture in situ or assessing of efflux from a few

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Address reprint requests to Dr. Miklós Gratzl, Department of Biomedical Engineering, Case Western Reserve University, Cleveland, OH 44106. Tel.: 216-368-6589; Fax: 216-368-4969; E-mail: mxg13@po.cwru.edu.

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cells or even a single cell is beyond the capabilities of existing technologies.

Simple and fast detection schemes, in principle, could also be realized by using the fact that doxorubicin (also called Adriamycin), which is one of the most widely used anticancer drugs in chemotherapy, and some other anticancer drugs are electroactive (Rao et al., 1978). Electrochemical methods with mercury electrode (Rao et al., 1978) and carbon paste electrode (Baldwin and Packett, 1981; Chaney and Baldwin, 1982, 1985) have been used to detect doxorubicin in phosphate buffer and patients' urine. The detrimental effect of mercury to live cells or the requirement of polishing the surface of a carbon paste electrode before each measurement, however, prevented these sensing schemes from being used for continuous cellular studies.

In the method described here, a carbon fiber (CF) microelectrode is used to monitor the efflux of doxorubicin from a monolayer of cells attached to a glass coverslip. The cell lines used were an auxotrophic mutant of Chinese hamster ovary cells, AUXB1, and its MDR subline, CH^RC5. The efflux rates obtained from the measured concentrations agreed well with reported results in the literature for both the drug-sensitive and drug-resistant cell lines studied.

This approach takes advantage of the microscopic cross section (7.5 μm in diameter) and good stability of electrodes made of carbon fiber, and the high sensitivity and sufficient resolution of adsorptive preconcentration followed by a reduction sweep, to monitor doxorubicin efflux from the same monolayer of preloaded cells in situ. The preconcentration step used in the measurement to adsorb doxorubicin to the surface of the CF electrode largely increases sensitivity to doxorubicin, extending the detection limit in phosphate-buffered saline (PBS) solution down to ~0.1 nM (Yi and Gratzl, 1993). The current temporal resolution (6 min/data) can be further improved to about one measurement per minute by decreasing the duration of the preconcentration period and narrowing the voltage range for the differential pulse voltammetry (DPV) scan. Further development of this technique can lead to virtually continuous monitoring of drug efflux from a few cells or even a single cancer cell and to efflux studies on other electroactive anticancer drugs (e.g., daunorubicin, chlorambucil, and fluorouracil).

MATERIALS AND METHODS

Materials

Carbon fiber was obtained from Zoltek Co. Pulled glass capillary tube (cat. no. 6010; A-M System) was used as the body of the CF electrode. Teflon-coated Ag wire (World Precision Instruments) and a 18-gauge 1½-inch stainless steel hypodermic needle (Becton-Dickinson) were used to prepare the reference and counter electrodes, respectively. The glass coverslip (Fig. 1 *A*) was custom-made, with a diameter of 4 cm and a thickness of 0.2 mm.

Crystalline doxorubicin (doxorubicin hydrochloride, Adria lot no. 89E07A; Fig. 2) was obtained from Adria Laboratories (Columbus, OH). The efflux medium contained minimum essential α medium (MEM α medium), 25 mM HEPES, and 10% fetal bovine serum, with a pH adjusted to 7.2–7.4. Powdered MEM α Medium and HEPES were obtained from GIBCO Laboratories (Grand Island, NY). Fetal bovine serum was purchased from Hyclone Laboratories (Logan, Utah).

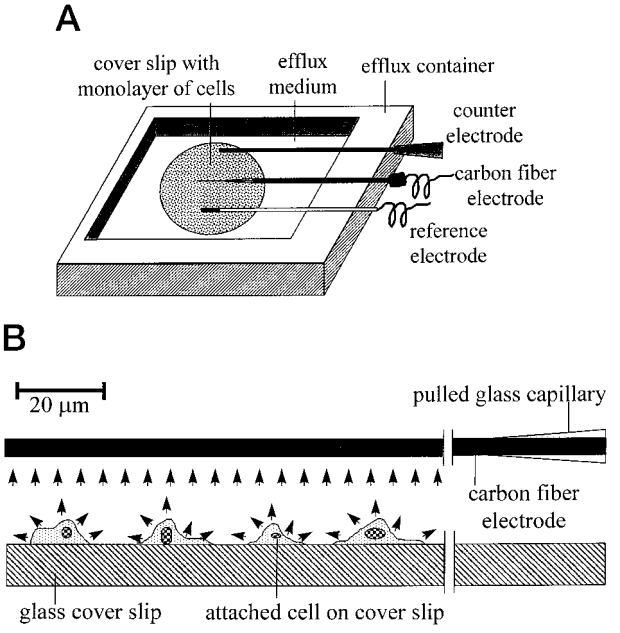

FIGURE 1 Experimental arrangement for doxorubicin efflux measurements. (*A*) The efflux container, made of plexiglas and covered by a plastic cover ensuring free air exchange between inside and outside the covered space (the cover is not shown here). The glass coverslip and the electrodes are under the efflux medium. The depth of the medium is ~1 mm. The electrodes are placed horizontally side by side, and the CF electrode is in the middle. The diameter of the coverslip is 4 cm. (*B*) Enlarged schematic diagram of the relative positions of the carbon fiber electrode and the effluxing cells. The arrows show the directions and patterns of diffusion of the effluxed doxorubicin.

Cell lines

The cell lines used in this study were an auxotrophic mutant of Chinese hamster ovary cells, AUXB1, and its MDR subline, CH^RC5. They were generous gifts from Dr. V. Ling (Ontario Cancer Institute, Toronto, Ontario, Canada); they were cultured in Dr. N. A. Berger's laboratory (Cancer Research Center, Case Western Reserve University, Cleveland, OH). The cells were cultured for 72 h to a density of 600-1000 cells/mm^2 in the same medium as the efflux medium at 37°C, on a glass coverslip with a diameter of 4 cm inside a petri dish, according to usual procedures (Daoud and Juliano, 1989).

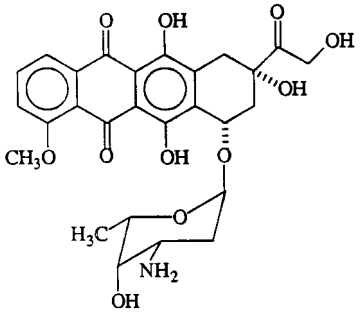

FIGURE 2 Chemical formula of doxorubicin, also called Adriamycin (Arcamone, 1981, p. 21).

Apparatus for electrochemical detection of doxorubicin

The electrochemical cell consisted of 1) a CF working microelectrode (diameter ~7.5 μm, exposed length 5 mm), 2) a Ag|AgCl reference electrode (diameter 0.3 mm, length 1 cm), and 3) a stainless steel counterelectrode, all immersed in the efflux medium (~5 ml) in the efflux container (Fig. 1 *A*). The electrodes were horizontally placed side by side with the CF electrode in the middle. The depth of 5 ml of medium with a cross-sectional area of 52 cm^2 in the efflux container was ~1 mm. The entire setup was kept inside the solid metal Faraday cage of the BAS 100A computer controlled potentiostat (Bioanalytical Systems) during measurements to protect the signal from electrical noise from the environment. The efflux container was partially covered by a plastic cover to prevent evaporation of the medium, but there was enough of an opening left at the edges of the cover for free air exchange between the inside and outside of the covered space. The electrodes were connected to the potentiostat with a PA-1 preamplifier. The data were transferred after each measurement via an RS-232 I/O interface to an Amiga-2000 computer for storage. This computer was also used for controlling the BAS 100A to execute preprogrammed experiments.

The CF microelectrode was fabricated as follows: 1) a long fiber (~12 cm) was sucked into a glass capillary by low vacuum; 2) the capillary was single pulled with a PB7 Narishige microelectrode puller; 3) the extending part of the fiber was cut to the appropriate length from the capillary tip with a pair of scissors; 4) the electrode body was filled with mercury, into which the stripped end (2 cm) of an insulated wire was inserted, to establish electrical contact with the CF electrode. This simple fabrication procedure provided a tight enough seal between the CF and the pulled tip of the glass capillary that no adhesive sealant had to be applied inside the capillary tip.

Measurement of doxorubicin concentrations close to the cell monolayer

A new CF electrode is used for every efflux experiment. The electrode is kept in situ throughout the experiment without any mechanical cleaning procedure. It is preconditioned in the efflux medium at 37°C for ~4 h just before the efflux experiment begins. The preconditioning protocol is the same as the measurement protocol. The protocol starts with a very negative potential (−1000 mV) applied to the CF electrode for 30 s, to electrochemically regenerate the electrode surface before each measurement. Then the electrode is kept open circuited for 5 min to preconcentrate doxorubicin at the CF surface by spontaneous adsorption. Finally, a DPV scan is performed from −300 to −1000 mV, which is the actual measurement step. This protocol is repeated every 6 min. The parameters of DPV in this entire work were 20-mV/s scan rate, 50-mV pulse amplitude, 50-ms pulse width, and 200-ms pulse period. Current sampling/averaging was done for 17 ms before the beginning and at the end of each pulse. Because in DPV differential current rather than current is recorded, $\Delta i/\Delta E$ values are reported in the figures and text. The voltage of the working CF electrode was measured and reported with respect to the Ag|AgCl reference electrode.

Before doxorubicin is added to the cell culture for incubation, the old culture medium is replaced by 5 ml fresh medium. The cells are then incubated for 1 h in this medium containing 6.4 μM doxorubicin at 37°C. After incubation, the coverslip with the monolayer of drug loaded cells is washed five times in ice-cold PBS solution (Kartner et al., 1983) within ~2 min and placed immediately after the last washing step into the efflux measurement container (Fig. 1 *A*) with 5 ml efflux medium. The CF electrode is placed horizontally just on top of the monolayer of the cells. Efflux measurements begin as soon as the coverslip is in the efflux container. The duration of the experiment is 1.5 h.

Calibration of the CF microelectrode

The temperature of the efflux medium during calibration is 37°C, and the same measurement protocol as the one used during the efflux experiment is applied to the CF microelectrode. The height of DPV peaks of the CF microelectrode is a log linear function of doxorubicin concentration over a broad range of concentrations, as shown in Fig. 3. The CF microelectrode used for the efflux measurements is calibrated after each efflux experiment in fresh efflux medium at 0.32 and 1.60 μM drug concentrations, respectively. These values are in the range of typical concentrations encountered in efflux experiments. The two obtained DPV peak heights determine the linear calibration to be used with respect to the logarithm of drug concentration. The values of the DPV peak height from the actual efflux measurement were then transformed to corresponding drug concentrations according to this two-point calibration. Because most deactivation of the CF electrode occurs during the first period of electrode stabilization and very little sensitivity loss occurs later, postcalibration can be used for the entire period of efflux measurement, without the need for extra corrections.

Normalization of the reconstructed drug efflux

To compare the results between experiments involving different cell densities on the coverslip, the obtained absolute efflux rates are divided by the density of the cells. The densities were calculated from a picture of the cell monolayer (1.3 mm × 0.9 mm) taken under microscope (Nikon Diaphot with 10× objective lens) just before each efflux experiment.

RESULTS

Because doxorubicin contains both a quinone and a hydroquinone functionality as shown in Fig. 2, it can be electrochemically reduced or oxidized. The carbonyl side chain can also yield a reduction current at very negative potentials (Rao et al., 1978). Two characteristic peaks related to doxorubicin were obtained with DPV in this work after adsorptive preconcentration of doxorubicin on the CF surface. One is a cathodic peak at about −630 mV, and another is an anodic peak around +250 mV. Both peaks are useful for analytical purposes. The positive potentials applied to obtain the anodic peak (especially those above +250 mV), however, tend to affect the surface of the CF electrode by generating oxidized surface groups (Edmonds, 1985), leading to insufficient sensitivity for efflux measurements after

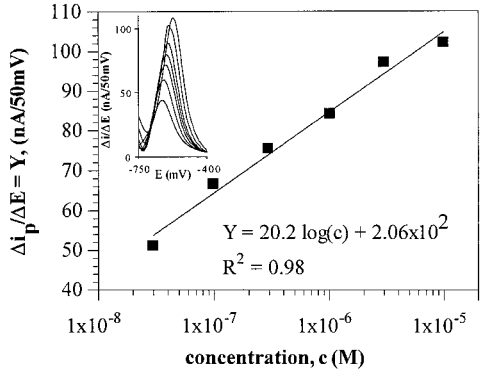

FIGURE 3 Calibration of a CF microelectrode for doxorubicin, using the "height," $\Delta ip/\Delta E$, of the cathodic DPV peaks at −630 mV versus Ag|AgCl in PBS solution. Peak heights were determined graphically and plotted against a logarithmic concentration axis. The concentration range here approximately overlaps with the one encountered in real efflux measurements near the cell monolayer. The inset shows the individual calibration sweeps, whose peak heights were used to construct the calibration curve.

a period of time. Using the cathodic peak, a more sensitive and stable protocol can be realized because of less damage done by the measurement itself to the active sites on the surface of the carbon fiber. Therefore, the cathodic peak at around −630 mV versus Ag|AgCl in PBS was used for the efflux measurements in this work. Typical DPV curves in the cathodic range are shown in Fig. 4. To keep the sensitivity of the CF electrode stable, the electrode had to be preconditioned in the same medium as the one used for the efflux measurement. Even when the cathodic peak is used, the sensitivity decreases to less than half of the original value after ~4 h of conditioning, with most of this change occurring during the first several measurement periods. Afterward, the sensitivity of the electrode remains relatively stable, with a 5% decrease per hour.

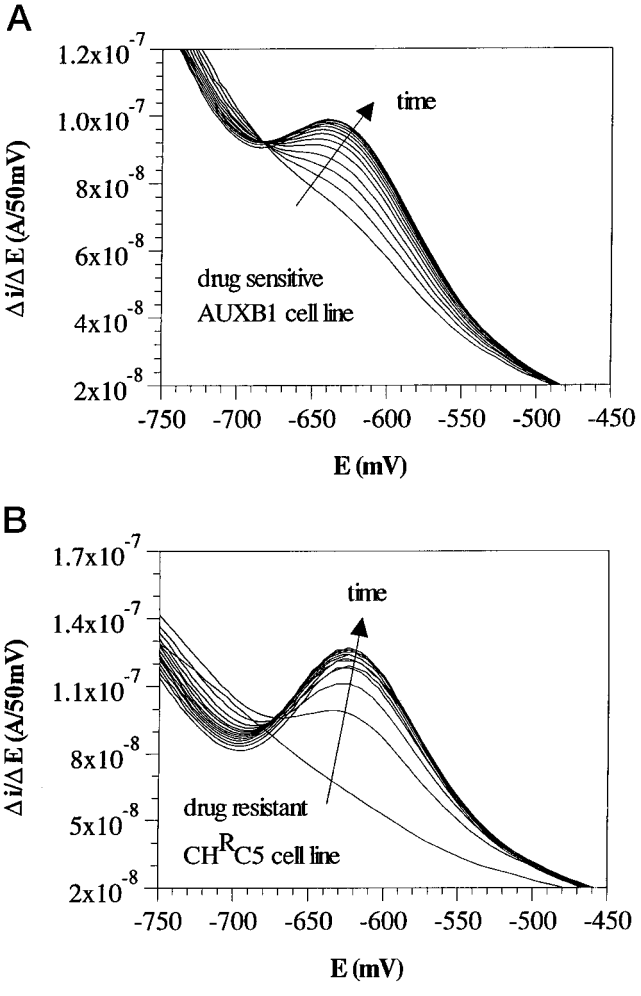

FIGURE 4 Results of drug concentration measurements above monolayers of sensitive AUXB1 and drug-resistant CH^RC5 cell lines in culture. Electrochemical regeneration of the carbon fiber microelectrode at −1000 mV for 30 s was followed by 5 min of preconcentration in open circuit conditions. The cathodic DPV peak at about −630 mV that was used was obtained with a scan from −300 to −1000 mV. The time interval between subsequent DPV scans is 6 min. The part of the DPV curves shown indicates the concentration above the monolayer of cells at different times. The arrows point in the direction of time increase. The increase in DPV wave height with respect to the baseline ($t = 0$) is proportional to the logarithm of local doxrubicin concentration. (*A*) Results from AUXB1 cell line. The cell density is 1.0×10^3 cells/mm^2. (*B*) Results from CH^RC5 cell line. The cell density is 6.2×10^2 cells/mm^2.

At higher doxorubicin concentrations (in the μM range and above) both diffusion and adsorption currents of doxorubicin can be discerned in both anodic and cathodic sweeps. When, however, the concentration of doxorubicin decreases to below 1 μM, it becomes very difficult to obtain a detectable signal originating from diffusion current at a CF electrode. The total doxorubicin concentration inside the preloaded cells can be 5–200 μM (Vichi and Tritton, 1992). The concentration is much lower outside the cells and decreases further as the CF electrode is placed further away from the cell monolayer during efflux experiments. One of the advantages of using a CF electrode is the spontaneous adsorption of doxorubicin to its surface, which dramatically increases the sensitivity of the measurement scheme when adsorption current is used as the signal. Surface coverage and, therefore, sensitivity can be increased by using a longer preconcentration period. Our studies showed that adsorption can be 97% complete after the 5-min preconcentration period, as used in the efflux measurement protocol in this work.

Contrary to the common practice of electrochemical cleaning of carbon fiber electrodes at high positive voltages, we found that a −1000-mV potential with a duration of 30 s effectively regenerated the surface of the carbon fiber before each subsequent measurement once the electrode was stabilized. This procedure created the same initial conditions for each adsorptive preconcentration step. (A positive potential higher than +300 mV can also regenerate the surface by oxidizing the drug, but it adversely affects the surface of the carbon fiber, leading to a gradual decrease in electrode sensitivity.) Reproducibility of the peak heights for the same concentration was typically 2–3% for a stabilized electrode with this cleaning protocol.

A typical calibration curve for doxorubicin is shown in Fig. 3, using the "heights" of cathodic DPV peaks as signal values. We found that this curve is log linear in the concentration range encountered in efflux studies ($r^2 = 0.98$, $n = 6$). We hypothesize that this is due to the logarithmic character of the adsorption isotherm of doxorubicin on carbon fiber within the concentration range studied.

Concentration changes near the monolayer of cells induced by efflux of doxorubicin from the cells have been monitored in this work by a CF electrode, using adsorptive preconcentration followed by DPV. Fig. 4 displays typical DPV curves obtained during efflux measurements for the AUXB1 and CH^RC5 cell lines, respectively. The time interval between subsequent DPV scans is 6 min. The arrows indicate the direction of time increase. The curves from both cell lines have a very clear and well-shaped peak around −630 mV, which corresponds to the position of one of the characteristic DPV peaks for doxorubicin. The peak becomes higher with longer efflux time for both resistant and sensitive cell lines at the beginning. The increase in DPV peak height with respect to the baseline ($t = 0$) is

proportional to the logarithm of actual local doxorubicin concentration.

The corresponding concentrations near the monolayer of cells versus time are shown in Fig. 5 *A* for both the drug-resistant and -sensitive cell lines. The dynamic characteristics for the two cell lines are different. The concentration above the drug-sensitive cells increases at the beginning and reaches a plateau after ~1 h. The measured concentration, however, did not begin to decrease during the experiment. The concentration above the drug-resistant cells increases faster than the one for the sensitive cell line and reaches a plateau after ~40 min. Then the concentration begins to decrease until the end of the experiment.

Diffusion of the drug from the cells into the efflux medium needs to be considered to reconstruct the efflux rate from the measured concentrations. To obtain initial efflux rates, we assumed that the efflux at the beginning, for the first three measurement periods (18 min), does not vary significantly. The efflux medium can be considered semi-infinite for such a short time period ($z \approx (DT)^{1/2} \ll h = 1$ mm, where h is the depth of the medium and z is the horizontal coordinate with the origin at the cell layer). With these assumptions, a relationship between the measured initial concentration time dependence and the efflux rate can be defined (Carslaw and Jaeger, 1959, p. 75):

$$c(t) = 2E\sqrt{t/\pi D} \tag{1}$$

where $c(t)$ is the measured concentration, E is the constant efflux density, D is the diffusion coefficient of doxorubicin in the efflux medium, and t is the time from the beginning of the experiment. Then efflux density can be obtained from $c(t)$ and t as follows:

$$E = 0.5c(t)\sqrt{\pi D/t} \tag{2}$$

If S is the cell density on the coverslip, then the normalized efflux rate for a single cell is

$$E_c = 0.5c(t)\sqrt{\pi D/t}/S \tag{3}$$

The initial three measured concentration values within ~18 min in Fig. 5 *A* have been used to calculate the average initial E_c for the sensitive and resistant cell lines, respectively. Cell densities were $\sim 1.0 \times 10^3$ cells/mm^2 for sensitive cells and 6.2×10^2 cells/mm^2 for resistant cells. With $D = 1.5 \times 10^{-6}$ cm^2/s (Shaw and Gratzl, unpublished observations), the initial efflux rate is $(3.1 \pm 0.3) \times 10^{-19}$ mol/cell · s for the drug-resistant cell line and $(6.6 \pm 0.3) \times 10^{-20}$ mol/cell · s for the sensitive cell line, as obtained from the initial three measurements in Figs. 4 and 5.

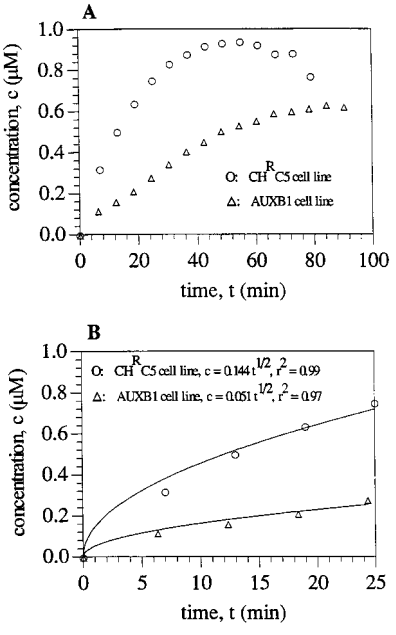

FIGURE 5 Measured concentrations near the monolayer of cells versus the efflux time. The DPV peak heights from Fig. 4 are used to calculate the concentrations with a semilogarithmic calibration as described in Materials and Methods. (*A*) Measured concentrations for the whole experiment period. (*B*) Enlarged initial part of *A*. The solid lines are curves fitted to the measured concentrations by the method of least squares according to Eq. 1.

DISCUSSION

The time interval between subsequent measurements was 6 min in this work, allowing for a period of doxorubicin preconcentration on the CF microelectrode of ~5 min. Adsorption reaches 97% completion after 5 min, and therefore it will not increase the signal significantly if longer measurement cycles are used. When it is necessary, however, much shorter preconcentration periods can also be selected, down to ~1 min. Then the relative surface coverage would become ~80% of the steady-state surface coverage, which would still provide enough sensitivity to measure the efflux of doxorubicin from cells.

A constant efflux rate was assumed in the above diffusion model for an initial period of 18 min to reconstruct efflux from the measured concentrations close to the monolayer of cells. This can be justified by the following observations: 1) The regression coefficients for the fitted curves in Fig. 5 *B* are 0.99 and 0.97, respectively. This means that the experimental data agreed well with the theoretical prediction (Eq. 1), and the assumption in the diffusion model is close to the real situation. 2) Because of the similarity between daunorubicin and doxorubicin, the efflux of doxorubicin across the cell membrane can be assumed to also consist of two components (Spoelstra et al., 1992): carrier-mediated transport approximated with a simple Michaelis-Menten-type kinetics and passive diffusion. It is reported (Miyamoto et al., 1990) that the amounts of drug remaining inside AUXB1 and CH^{R}C5 cell lines after 18 min are ~90% and ~25% of the initial amounts, respectively. For the sensitive cells, the dominant efflux process is passive diffusion, and therefore intracellular drug content decreases by only ~10% during the initial 18 min. Thus it is reasonable to assume the efflux rate to be constant during this period. For the CH^{R}C5 cell line, the error of the estimated initial efflux rate induced by assuming an initially constant efflux is

expected to be larger than for the AUXB1 cell line, because a larger drop in intracellular drug content occurs for the resistant cells during the same period. The standard deviation of the efflux rate obtained from the initial three measured concentrations with Eq. 3 is, however, just ~10% of the mean estimated efflux. This means that efflux stays relatively constant during the initial 18 min, even in the resistant cell line. This also implies that if the dominant efflux process in CH^RC5 is active transport, then the Michaelis constant, K_M, is lower than the intracellular drug concentration during most of the initial 18 min.

The concentration changes observed with the new microelectrode technique during the entire experimental period can also be rationalized. The concentrations increase for both cell lines at the beginning. This indicates a significant efflux of doxorubicin from the cells. After ~40 min from the beginning, the concentration above the resistant cell line reaches a maximum and then begins to decline. This means a decrease or a halt of efflux. (It should be noted that although the solution layer above the cells is thin, it can be considered as a semiinfinite medium for the given D value and time span of each experiment. This rationalizes that once the efflux rate decreases or halts, the local concentration at the CF electrode also begins to decrease.) The behavior of the drug-sensitive cell line is different during this later period: the concentration increases and then reaches a plateau, which implies a relatively steady efflux of doxorubicin.

These results are consistent with reported results for these two cell lines (Miyamoto et al., 1990). This finding validates the electrochemical efflux measurement technique for drug resistance studies as introduced in this work.

There are about four to seven cells under a 100-μm-long segment of carbon fiber, as shown in Fig. 1 *B*. Because the fiber diameter is ~7.5 μm, which is smaller than that of the cells, the number of cells effectively contributing to the signal measured by a 100-μm-long carbon fiber is about three to five cells, if diffusion is mainly one-dimensional. Some nonuniformities in efflux rates by individual cells are likely to be present, but they even out over distances similar to the electrode length. Thus unidirectional diffusion away from a uniform planar source is a reasonable approximation, assuming that perturbation of the drug concentration distribution by the CF microelectrode itself is negligible.

The average signal increase per 6 min measured with a 5-mm-long fiber is ~10 nA/50 mV, as shown in Fig. 4. Then signal change due to efflux from an average single cell should be ~50 pA/50mV for every 6 min. Signals of this magnitude are still accessible by microvoltammetry. This infers that it may be feasible to detect drug efflux from a single cancer cell with the technique proposed in this work, by using a 20-μm-long cylindrical electrode or a microdisk CF electrode placed close to the effluxing single cell.

Preliminary results obtained with a CF microdisk electrode (Lu and Gratzl, manuscript in preparation) indeed proved that efflux from both single AUXB1 and CH^RC5 cells can be monitored with the technique introduced in this work. This is important because heterogeneities in efflux characteristics within one cell type (sensitive or drug-resistant line) cannot be characterized without a single-cell efflux measurement scheme. The technique is also ideally suited to obtaining efflux data of high temporal resolution from the beginning of efflux, which renders determination of the parameters of both passive and active efflux numerically better conditioned than when only techniques of low temporal resolution are available.

It is reported that daunorubicin, marcellomycin, chlorambucil, fluorouracil, and methotrexate are also electroactive (Rao et al., 1978; Chastel et al., 1989; Wang et al., 1987; Temizer and Onar, 1988). Therefore, it is likely that efflux of these drugs from populations as well as single cancer cells can also be studied with this new scheme.

Voltammetry of doxorubicin at a CF microelectrode, as described in this work, is simple to perform and has excellent analytical characteristics for cell culture studies. The advantages of this method over conventional approaches are 1) It can be used to continuously monitor the same cell population. 2) It uses electrochemical characteristics that may be the only chance for in situ monitoring when the drug is nonfluorescent. 3) For continuous single-cell-level drug efflux studies, only this technique is currently available.

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REFERENCES

Arcamone, F. 1981. Doxorubicin: Anticancer Antibiotics. Academic Press, New York.

Astier, A., B. Doat, M. J. Ferrer, B. Benoit, and R. Leverge. 1988. Enhancement of adriamycin antitumor activity by its binding with an intracellular sustained-release form, polymethacrylate nanospheres, in U-937 cells. *Cancer Res.* 48:1835–1841.

Baldwin, R. P., and D. Packett. 1981. Electrochemical behavior of adriamycin at carbon paste electrodes. *Anal. Chem* 53:540–542.

Bradley, G., P. F. Juranka, and V. Ling. 1988. Mechanism of multidrug resistance. *Biochim. Biophys. Acta,* 948:87–128.

Carslaw, H. S., and J. C. Jaeger. 1959. Conduction of Heat in Solids. Clarendon Press, Oxford.

Chaney, E. N., Jr., and R. P. Baldwin. 1982. Electrochemical determination of adriamycin compounds in urine by preconcentration at carbon paste electrodes. *Anal. Chem.* 54:2556–2560.

Chaney, E. N., Jr., and R. P. Baldwin. 1985. Voltammetric determination of doxorubicin in urine by adsorptive preconcentration and flow injection. *Anal. Chim. Acta.* 176:105–112.

Chastel, O., J. M. Kauffmann, and G. J. Patriarche. 1998. Hydrophobic stripping voltammetry using a lipid-modified glassy carbon electrode. *Anal. Chem.* 61:170–173.

Daoud, S. S., and R. L. Juliano. 1989. Modulation of doxorubicin resistance by valinomycin (NSC 122023) and liposomal valinomycin in Chinese hamster ovary cells. *Cancer Res.* 49:2661–2667.

Edmonds, T. E. 1985. Electroanalytical applications of carbon fiber. *Anal. Chim. Acta.* 175:1–22.

Gottesman, M. M., C. A. Hrycyna, P. V. Schoenlein, U. A. Germann, and I. Pastan. 1995. Genetic analysis of the multidrug transporter. *Annu. Rev. Genet.* 29:607–649.

Inaba, M., H. Kobayashi, Y. Sakurai, and R. K. Johnson. 1979. Active efflux of daunorubicin and adriamycin in sensitive and resistant sublines of P388 leukemia. *Cancer Res.* 39:2200–2203.

Kartner, N., M. Shales, J. R. Riordan, and V. Ling. 1983. Daunorubicin-resistant Chinese hamster ovary cells expressing multidrug resistance and a cell-surface P-glycoprotein. *Cancer Res.* 43:4413–4419.

Krishan, A. 1990. Rapid determination of cellular resistance-related drug efflux in tumor cells. *In* Methods in Cell Biology, Vol. 33, Flow Cytometry. Z. Daraynkiewicz and H. A. Crissman, editors. Academic Press, San Diego.

Krishan, A., and R. Ganapathi. 1980. Laser flow cytometric studies on the intracellular fluorescence of anthracyclines. *Cancer Res.* 40:3895–3900.

Marquardt, D., and M. S. Center. 1992. Drug transport mechanisms in HL60 cells isolated for resistance to adriamycin: evidence for nuclear drug accumulation and redistribution in resistant cells. *Cancer Res.* 52:3157–3163.

Michelson, S., and D. Slate. 1994. A mathematical model for inhibition of the multidrug resistance associated P-glycoprotein pump. *Bull. Math. Biol.* 56:207–223.

Miyamoto, Y., T. Oda, and H. Maeda. 1990. Comparison of the cytotoxic effects of the high- and low-molecular-weight anticancer agents on multidrug-resistant Chinese hamster ovary cells in vitro. *Cancer Res.* 50:1571–1575.

Nooter, K., H. Herweijer, R. R. Jonker, and G. Van Den Engh. 1990. On-line flow cytometry—a versatile method for kinetic measurments. *In* Methods in Cell Biology, Vol. 33, Flow Cytometry. Z. Daraynkiewicz and H. A. Crissman, editors. Academic Press, San Diego.

Rao, G. M., J. W. Lown, and J. A. Plambeck. 1978. Electrochemical studies of antitumor antibiotics. III. Daunorubicin and adriamycin. *J. Electrochem. Soc.* 125:534–539.

Spoelstra, E. C., H. Dekker, G. J. Schuurhuis, H. J. Broxterman, and J. Lankelma. 1991. P-Glycoprotein drug efflux pump involved in the mechanisms of intrinsic drug resistance in various colon cancer cell lines. *Biochem. Pharmacol.* 41:349–359.

Spoelstra, E. C., H. V. Westerhoff, H. Dekker, and J. Lankelma. 1992. Kinetics of daunorubicin transport by P-glycoprotein of intact cancer cells. *Eur. J. Biochem.* 207:567–579.

Temizer, A., and A. N. Onar. 1988. Determination of methotrexate in human blood plasma by adsorptive stripping voltammetry. *Talanta.* 35:805–806.

Vichi, P., and T. R. Tritton. 1992. Adriamycin: protection from cell death by removal of extracellular drug. *Cancer Res.* 52:4135–4138.

Wang, J., M. S. Lin, and V. Villa. 1987. Investigation of the adsorptive stripping voltammetric behaviour of the anticancer drugs chlorambucil and 5-fluororacil. *Analyst.* 112:247–251.

Yi, C., and M. Gratzl. 1993. Trace analysis of the anticancer drug adriamycin using carbon fiber microvoltammetry for single cell drug resistance studies. *Current Separations.* 12:124.