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Electrochemical Monitoring of Rat Mammary Adenocarcinoma Cells: An *In Vitro* Assay for Anticancer Drug Selection

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Abstract: We report the application of an electrochemical biosensor (Oncoprobe™; Marks & Clerk, Manchester, UK) to determine whether changes in the open circuit potential (OCP) of rat mammary adenocarcinoma cells (MTLn3) treated *in vitro* with four cytotoxic anticancer drugs could predict their effects *in vivo*. MTLn3 cells were seeded onto sensors, then exposed to each anticancer compound (cisplatin, doxorubicin, paclitaxel, or vinblastine), and monitored for 44 hours. Electrochemical monitoring *in vitro* detected OCP responses to all four drugs, with cisplatin and doxorubicin producing greater changes over a shorter period than vinblastine and paclitaxel. Syngeneic MTLn3 cells were used to generate palpable tumors in 50 female Fischer 344 rats. Animals were divided into five equal groups; on day 12 four of the groups received an anticancer drug, and one received a saline control. Fourteen days later the animals were killed, and primary tumor weights were determined. Tumors from cisplatin- and doxorubicin-treated rats were significantly reduced in weight compared to the control, paclitaxel-, and vinblastine-treated groups. The anticancer drug-induced changes observed through real-time electrochemical monitoring of MTLn3 cells *in vitro* correlated well with the *in vivo* animal model, unlike the conventional end-point assays of lactate dehydrogenase release and Alamar Blue.

Introduction

We have previously reported the principles of an electrochemical biosensor, Oncoprobe (Marks & Clerk, Manchester, UK), for passively recording the open circuit potential (OCP) of confluent cell monolayers on gold sensors and its application in identifying anticancer drug-resistant ovarian carcinoma cell lines *in vitro*.[1,2] Electrochemical potentials are generated at the surface of living cells through cellular processes driving reducing/oxidizing (redox) reactions and inducing ionic gradients.[3,4] This technology has now been developed into a disposable, miniaturized, multiwell biosensor system with potential applications in drug discovery and theranostics (manuscript in preparation).

Chemosensitivity testing is the process whereby a patient's cancer cells or tissues are screened for an effective anticancer treatment, either by directly exposing the cells to the compounds or through analysing genetic signatures.[5,6] The American Society of Clinical Oncology performed an assessment of chemotherapy sensitivity and resistance assays in 2004 and concluded that their use was not recommended outside of the clinical trial setting.[7] In that assessment, methods such as methyl thiazolyl-diphenyl-tetrazolium bromide, differential staining cytotoxicity, ATP bioluminescence, and [^{3}H]thymidine incorporation assays were considered. Since the American Society of Clinical Oncology assessment, chemosensitivity studies have continued to be carried out using these techniques as well as other methods, such as the collagen gel droplet-embedded culture drug-sensitivity test, Alamar Blue assay, and automated procedures such as DIMSCAN, a fluorescence-based digital imaging system using fluorescein diacetate to detect cell viability.[8–10]

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ABBREVIATIONS: LDH, lactate dehydrogenase; OCP, open circuit potential.

These methods are largely dependent upon defining an end-point at which the assay is carried out, unlike the Oncoprobe system described here, in which electrochemical potentials are monitored in real-time throughout the course of an experiment.

Similarly, other whole-cell biosensors have been developed to exploit the apparent deficiencies of conventional end-point assays in their application to chemosensitivity testing. Such technologies employed include impedance, optical, and quartz crystal microbalance detection methods.[11–14]

In this study we demonstrate the potential of electrochemical monitoring for the selection of anticancer drugs using the MTLn3 rat mammary carcinoma model.[15–17] Moreover, we subsequently compare the data obtained from the *in vitro* electrochemical assay with two recognized end-point chemosensitivity assays (*viz*., lactate acid dehydrogenase [LDH] and Alamar Blue).

Materials and Methods

Oncoprobe apparatus

The Oncoprobe biosensor unit comprises an eight-well culture assembly featuring a reusable acrylic culture vessel with disposable ceramic probe attached to an analytical monitor. The ceramic probe is sandwiched into the culture assembly with silicon O-ring seals and secured with M3 bolts. Each well contains three 1-mm-diameter gold sensors around a central silver/silver chloride reference electrode and was produced using screen-printed conductive inks (DuPont, Bristol, UK). Electrodes are connected by gold tracking to the edges with a dielectric insulating layer printed above. Gold-plated spring-loaded contacts (Coda Systems, Halstead, UK) interface the probe and analytical monitor via a bridging printed circuit board embedded into the culture assembly. Silicon O-rings maintain an atmospheric seal between the analytical monitor and cell culture assembly. The analytical monitor was designed and manufactured by Uniscan Instruments Ltd. (Buxton, UK). Biocompatibility of cells with all components of the bioassay device was tested, particularly the adherence of cells to the gold substratum. Electrochemical OCPs generated at the cell:sensor interface are measured and processed by the analytical monitor and then displayed in real-time via proprietary software on a personal computer. A sampling frequency of 10 min was employed for all the experiments. The Oncoprobe biosensor unit operates within an incubator at 37°C with an atmosphere of 5% CO_2 in humidified air.

Cell cultures

Viable cell suspensions of rat mammary adenocarcinoma (MTLn3) cells were seeded onto sterile sensors in Minimal Essential Medium, α modification with ribonucleosides and ribonucleotides containing 10% fetal calf serum and antibiotic/antimycotic (all from Gibco, Paisley, UK) at a density of 2×10^5 cells per well. After seeding, the cells settled, attached, and spread overnight. Parallel cultures at identical relative cell densities were established in eight-well Nunc Labtek II culture slides in order to assess the degree of confluence and viability microscopically and in 96-well plates (all from VWR International, Lutterworth, UK) for Alamar Blue and LDH end-point assay experiments.

For scanning electron microscopy, the MTLn3 cells were cultured overnight on the three different substrata (silicon chip, bare ceramic probe, and screen-printed gold electrode), fixed in phosphate-buffered 3% glutaraldehyde, and processed and viewed as described.[2]

Anticancer drug experiments

Stock solutions of doxorubicin, cisplatin (Pharmacy, Wythenshawe Hospital, Manchester, UK), vinblastine, and paclitaxel (Sigma, Poole, UK) were used to prepare working dilutions in Minimal Essential Medium, α modification containing 2% fetal calf serum for electrochemical monitoring or 0.2% lactalbumin hydrolysate (Sigma) for end-point assays. Cell cultures on Oncoprobe sensors, chamber slides, and 96-well plates were treated with the same concentrations of each drug and an appropriate control. Single drug concentrations were chosen that represented clinically relevant concentrations, as judged by previous *in vitro* studies.[18,19] The electrochemical potential of the cells was then monitored at a 10-min frequency for 24 h using the Oncoprobe system ($n = 8$). In selected experiments the medium was replenished and monitored for a further 20 h ($n = 6$).

Chamber slides containing treated and untreated control cells were fixed with 70% ethanol after 24 h, air-dried, and subsequently stained with Harris' hematoxylin (Sigma). Slides were viewed and micrographs taken with a Motic Instruments (Ipswich, Suffolk, UK) DM-BA300 digital photomicroscope system.

In vivo *experiments*

MTLn3 cells, originally derived from 13762NF mammary adenocarcinoma lung metastases, were used to generate palpable tumors in a mammary fat pad of 50 female Fischer 344 rats.[15,17] Animals were divided into five groups of 10 rats, each of which received a 100-μl tail-vein injection at day 12 as follows: group A, saline; group B, cisplatin (3.3 m*M*); group C, doxorubicin (3.7 m*M*); group D, paclitaxel (7.0 m*M*); and group E, vinblastine (0.3 m*M*). Drug concentrations relative to rat body weight (approximately 150 g) were selected from recommended intravenous treatments as used for cancer patients. After a further 14 days the animals were killed, and weights of the primary tumor were determined. This protocol had received institutional approval by the Animal Care and

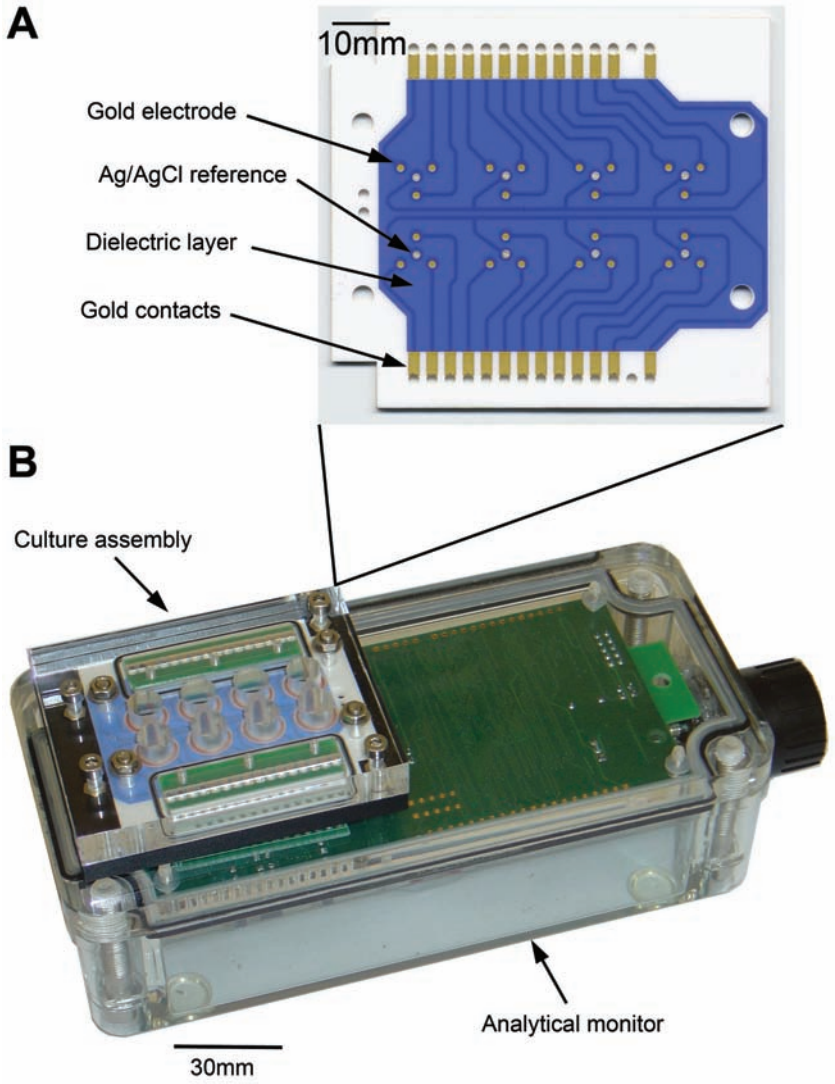

FIG. 1. Oncoprobe instrument. (**A**) Disposable ceramic probe featuring gold sensors/tracking/contacts, Ag/AgCl reference electrode, and dielectric insulation, as described in Materials and Methods. (**B**) Analytical monitor with the eight-well cell culture assembly interfaced.

Use Committee of the University of Tennessee Health Science Center, Memphis, TN.

Since all the drug-treated animals showed no adverse effects on the single drug administration at day 12 (as judged by final total body weight), a second *in vivo* experiment was performed, essentially as described above, but applying the same drug treatments on days 12 and 19, respectively, before sacrifice at day 25. The results of that study were qualitatively similar to those described.

End-point assays

Alamar Blue (Serotec, Oxfordshire, UK) and LDH (Sigma) assays were carried out in 96-well plates at the 24-h end-point according to the manufacturer's instructions. In brief, Alamar Blue was added at a 1:10 ratio to each well and incubated for 3 h; the reduction in absorbance at 600 nm was then measured spectrophotometrically. Similarly, the relative levels of LDH released were determined by assaying 200 μl of medium with a tetrazolium substrate–NADH complex yielding formazan and reading the absorbance at 450 nm. Total LDH levels were obtained through lysis of cell cultures. Phenol red was excluded from the medium in these assays in order to maximize sensitivity, and appropriate blanks were used for each group.

Results

Electrochemical monitoring in vitro

The Oncoprobe cell assembly for receiving the MTLn3 cells is shown in Fig. 1 and is described in detail in Materials and Methods.

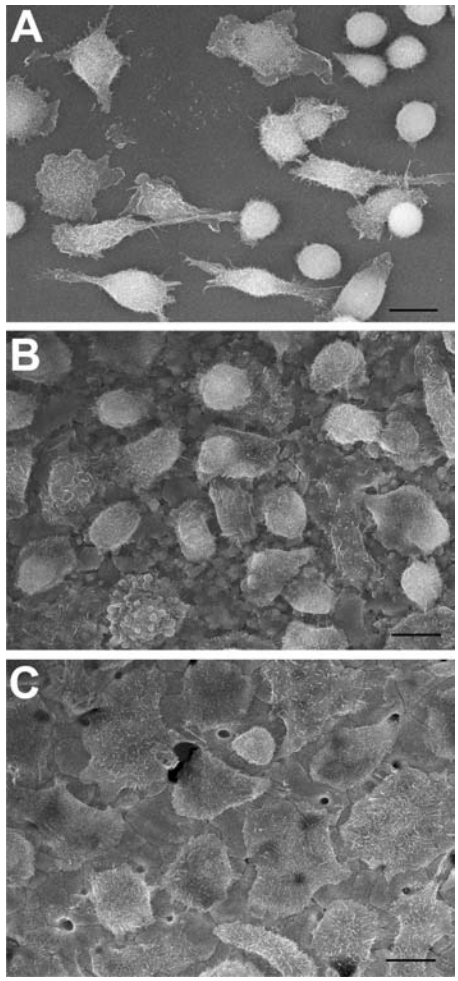

FIG. 2. Scanning electron micrographs of MTLn3 cells on (**A**) silicon chips, (**B**) bare ceramic probe, and (**C**) screen-printed gold ink surface. Scale bar = 10 μm. (Courtesy of Stephen Murray, Paterson Institute for Cancer Research, Manchester, UK.)

The morphology and adherence of MTLn3 cells on the gold electrodes are demonstrated by the scanning electron micrographs in Fig. 2. Although the surface of the printed conductive gold ink electrode shows occasional pits (Fig. 2C) compared to the polished silicon chip (Fig. 2A), it is more uniform than the underlying ceramic base (Fig. 2B); all substrata show the adherence of cells and effective biocompatibility to each surface. Figure 3 illustrates the effect of the four anticancer drugs on the OCP of MTLn3 cells *in vitro*. Approximately 12 h after treatment the OCP of control and paclitaxel- and vinblastine-treated wells starts to increase, whereas the OCP signal begins to reduce in the cisplatin- and doxorubicin-treated cells. By 24 h of real-time monitoring it is clear that the cisplatin and doxorubicin treatments yield a much reduced OCP signal compared to the paclitaxel- and vinblastine-treated cells (Fig. 3A). After 24 h the medium was carefully replenished without drugs, and monitoring was continued for a further 20 h. During this second part of the experiment cisplatin- and doxorubicin-treated cells continue to show low OCP values, with vinblastine and paclitaxel treatments also showing reduced OCP values that begin to recover from 42 h (Fig. 3B).

Microscopy

After 24 h, micrographs of cells treated with each of the anticancer compounds (Fig. 4) all display some morphological effects associated with apoptosis. Compared to the untreated cells (Fig. 4A) the doxorubicin-treated cells (Fig. 4C) appear to show signs of cell shrinkage and condensing of DNA, relatively early events in the cycle of programmed cell death, whereas cisplatin-, paclitaxel-, and vinblastine-treated cells all display symp-

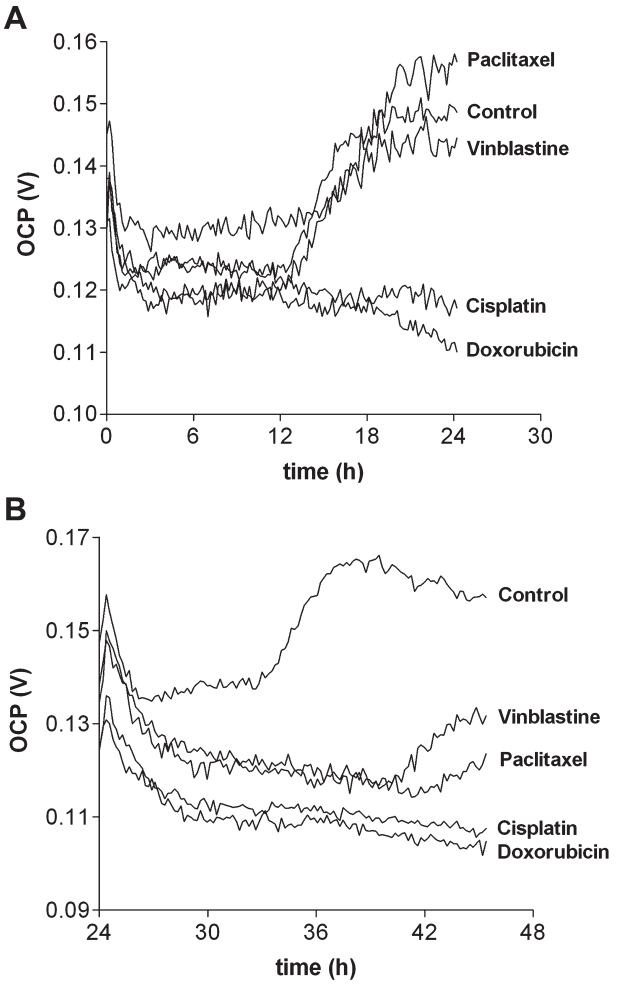

FIG. 3. (**A**) Real-time electrochemical OCP of MTLn3 cells treated with cisplatin (50 μM), doxorubicin (20 μM), paclitaxel (1 μM), and vinblastine (500 nM) anticancer drugs compared with untreated controls after 24 h. (**B**) Following replenishment with drug-free medium, monitoring was continued for a further 20 h.

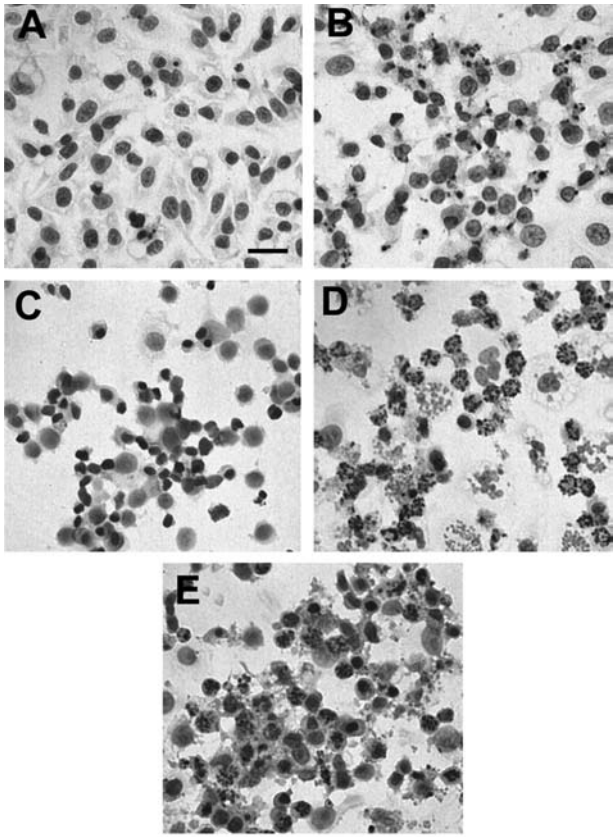

FIG. 4. Photomicrographs of MTLn3 cells stained with Harris' hematoxylin after 24 h of treatment with (**A**) control, (**B**) cisplatin (50 μM), (**C**) doxorubicin (20 μM), (**D**) paclitaxel (1 μM), and (**E**) vinblastine (500 nM). Scale bar = 20 μm.

toms of the later stages of apoptosis such as pyknotic nuclei and fragmented DNA. Although the micrographs in Fig. 4 suggest a reduction in cell numbers due to anticancer drug treatments, this largely reflects the result of the histological staining procedure rather than the confluent cell layers observed by phase-contrast microscopy in the parallel chamber slide (data not shown). Any compromised cells that remain partially attached to the surface are not retained, whereas in the Oncoprobe system such cells would continue to be monitored and generate an OCP, which would concur with the reduction in OCP observed in Fig. 3B after replenishment of cell culture medium.

In vivo *animal data*

Figure 5A shows two representative primary tumors excised from each of the five groups of rats. Body weights of each animal were taken at day 11 before drug injection and again at day 25. All drug-treated animals progressed to day 25. The average tumor burden, as judged by wet weight of the excised primary tumor, of each group of animals determined 14 days after treatment is shown in Fig. 5B. Of the four anticancer drug treatments only cisplatin and doxorubicin show a significant response by Student's t test ($P < 0.05$) versus the control group.

End-point cytotoxicity assays

To demonstrate the advantages of the electrochemical methodology over established chemosensitivity assays, a comparative study was made using the conventional LDH and Alamar Blue assays. The percentage of total LDH released into the medium (an indication of cytolysis/cell death) after 24 h is shown in Fig. 6A. Each anticancer drug treatment results in an increased release of LDH into the medium relative to the untreated control, with doxorubicin treatment releasing considerably more LDH than the other three drugs, which are comparable. Similarly, the Alamar Blue assay (Fig. 6B), reportedly an assessment of metabolic activity, demonstrates a reduction in absorbance values in all the treated cultures versus the untreated control. However, unlike the LDH assay, the four treatments do not significantly differ, with each showing a similar reduction in absorbance versus the control. Thus, both assays fail to predict the relative effectiveness of these anticancer drugs on the MTLn3 cells *in vivo*, unlike the electrochemical data presented in Fig. 3A.

Discussion

This study has attempted to demonstrate whether *in vitro* electrochemical responses by rat mammary carcinoma (MTLn3) cells to four anticancer drugs would effectively predict subsequent *in vivo* results. All four drugs were shown to affect the electrochemical OCP generated by the MTLn3 cells *in vitro*. Within the first 24 h of monitoring, doxorubicin and cisplatin were the most effective at reducing the OCP relative to the untreated control values. Paclitaxel and vinblastine also induced an OCP response, but only after 24 h, with cells receiving those treatments showing signs of recovery from 36 to 40 h. These *in vitro* results showed a good correlation with the *in vivo* animal data, in which doxorubicin and cisplatin markedly reduced the tumor burden.

We recognize there are several aspects in this study that might be investigated further; examples include the *in vitro* drug concentrations and duration of their exposure to cells, the number and timing of tail-vein injections for each animal, and termination point for the *in vivo* study. A particularly important aspect of this study was in the choice of an appropriate *in vitro* anticancer drug concentration. There is no simple correlation between the concentration of compounds used in cell culture conditions and that typically experienced at the tumor site within a patient.[20] Peak plasma concentrations,

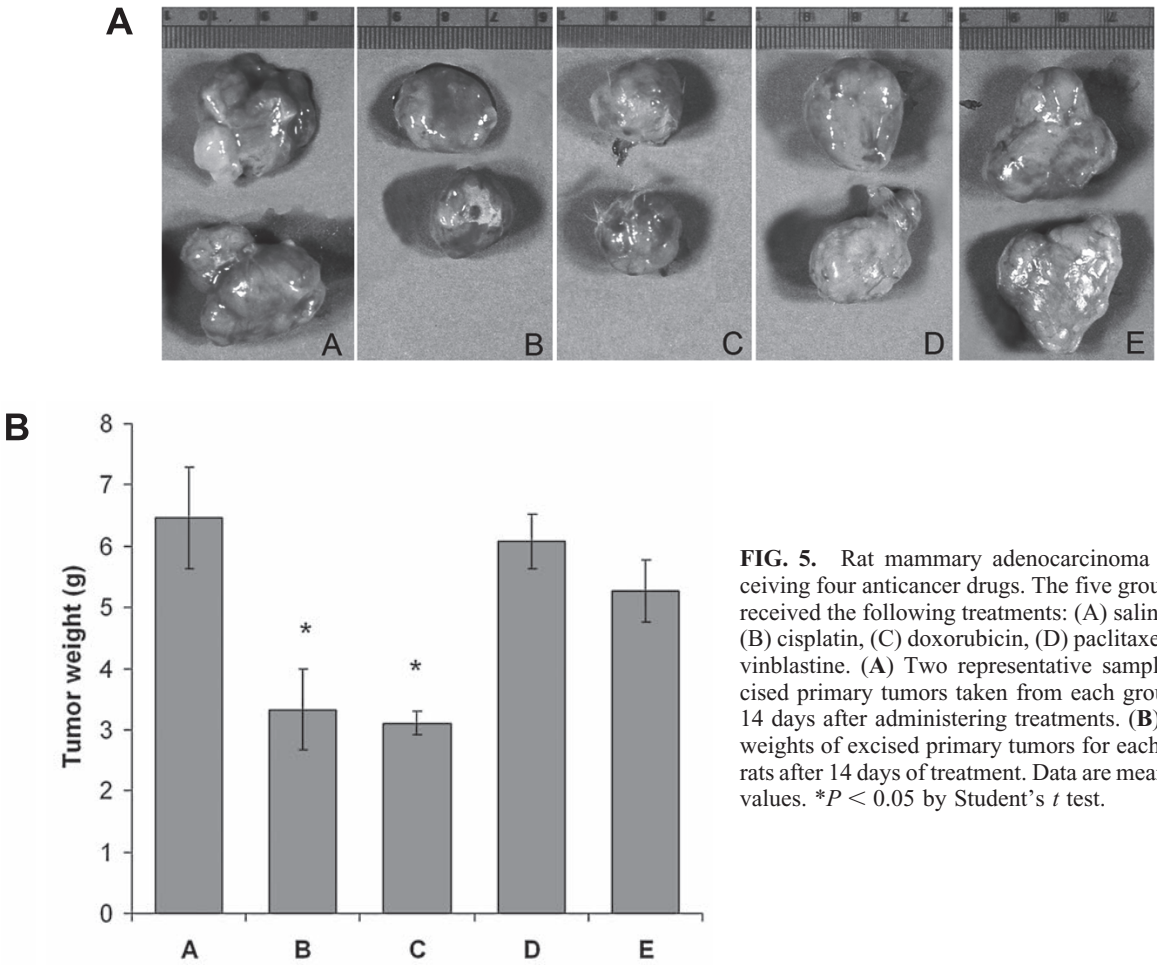

FIG. 5. Rat mammary adenocarcinoma study receiving four anticancer drugs. The five groups of rats received the following treatments: (A) saline control, (B) cisplatin, (C) doxorubicin, (D) paclitaxel, and (E) vinblastine. (**A**) Two representative samples of excised primary tumors taken from each group of rats 14 days after administering treatments. (**B**) Average weights of excised primary tumors for each group of rats after 14 days of treatment. Data are mean ± SEM values. $*P < 0.05$ by Student's *t* test.

which have been used as a reference point in drug resistance studies, are not appropriate in determining levels of chemosensitivity.[21] For example, penetration of doxorubicin through tissues has been shown to be particularly poor, with the drug binding to DNA and being sequestered into endosomes within cells surrounding the vasculature.[20] Therefore, the use of doxorubicin peak plasma concentrations *in vitro* would not accurately reflect the dose experienced within a patient's tissues. In this study, the drug concentrations employed *in vitro* were those previously shown either to effectively induce apoptosis in MTLn3 cells or to be relevant to a therapeutic dose *in vivo*.[18,19]

To demonstrate the advantages of the real-time electrochemical methodology over established chemosensitivity assays, MTLn3 cells were subjected to the same drug treatments for 24 h before carrying out assays for LDH release and Alamar Blue reduction. Both assays showed differences between treated and untreated cell cultures. However, with the exception of the LDH assay data for doxorubicin there was little variation in the assay results for the four treatments. When such data were related to the animal study it was clear that a complete correlation with *in vivo* results was not possible. By contrast, OCP values at 24 h clearly showed the subsequent benefits of cisplatin and doxorubicin treatments. Moreover, whereas the real-time electrochemical bioassay was monitored under normal cell culture conditions, both endpoint assays required modifications such as the elimination of phenol red or serum—components known to affect the sensitivity of those assays, which may also affect cell behavior *in vitro*.

Although the photomicrographs of MTLn3 cells exposed to the four anticancer drugs (Fig. 4) might suggest that detachment of cells has caused the reduced OCP values recorded in Fig. 3, phase-contrast microscopy of those cultures, prior to fixation and histological staining, showed confluent cells, as previously demonstrated for cisplatin-treated A2780 ovarian carcinoma cells.[2] Electrochemical monitoring after withdrawing the treatments (Fig. 3B) reproduces to some degree the effects of histological processing shown in Fig. 4, in that the OCP of paclitaxel- and vinblastine-treated cells is reduced. However, the use of real-time electrochemical monitoring al-

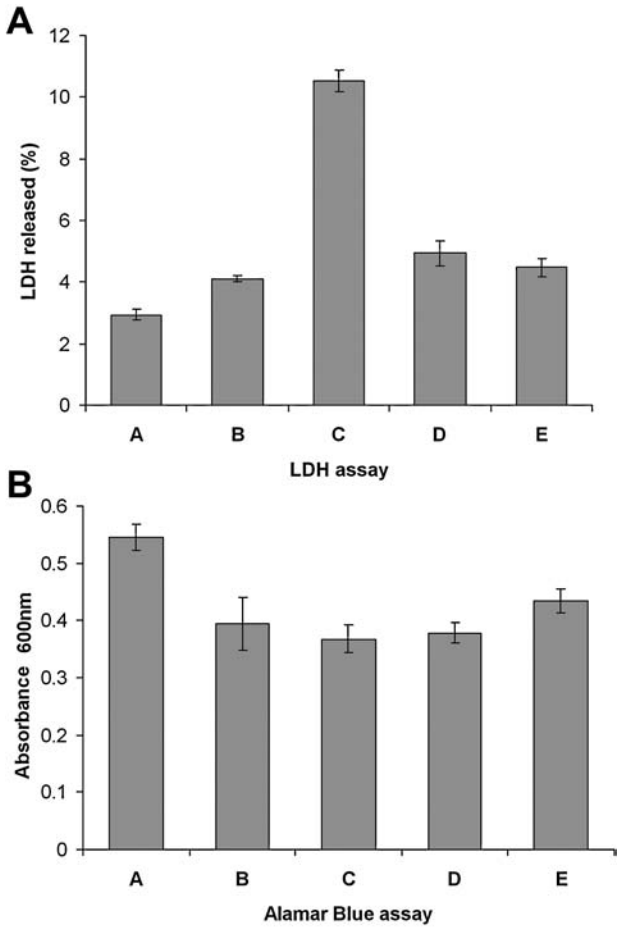

FIG. 6. (**A**) LDH and (**B**) Alamar blue assay data after 24 h of drug treatment: saline control (A), cisplatin (B), doxorubicin (C), paclitaxel (D) and vinblastine (E). Data are mean ± SD values ($n = 5$).

lows the recovery of the OCP values for the paclitaxel- and vinblastine-treated cells, unlike that of cisplatin and doxorubicin treatments.

The electrochemical responses generated by MTLn3 cells differ from the human cancer cell lines on which we have previously reported,[1,2] in that the OCP of controls increases during the course of the experiment. Usually, after a period of settling and attachment, a confluent monolayer of cells on an Oncoprobe sensor would reach a stable OCP plateau, at which point an anticancer drug or other treatment would be applied. However, because of the high metabolic and proliferative rate of MTLn3 cells,[22] proliferation continues beyond the normal confluent state. This overgrowth drives the OCP value higher and eventually results in medium depletion, which is reflected electrochemically by a reduction in OCP. In order to limit this effect, a subconfluent density of cells was seeded that would reach confluence during the experiment but not limit the response of the assay.

As previously stated, other whole-cell biosensors have been developed for the purpose of chemosensitivity and resistance testing. To date, one of the best-established technologies is that of electrical cell substrate impedance sensing.[23,24] It is a system whereby a voltage is applied to the culture and the resistivity of the cells is calculated from the current measured. While there would appear to be similarities in the Oncoprobe system to electrical cell substrate impedance sensing, the electrochemical monitoring method employed in the Oncoprobe system passively measures the electrochemical potential generated by cells in contact with gold sensors versus a reference electrode, this being largely generated through redox reactions driven by cellular processes.

We have previously described some of the contributing factors that generate the OCP signal at the interface between the gold sensor and the cell surface.[1,2] While we recognize that some micromodification of the sensor surface might encourage improvements in cell adhesion and OCP signal, possibly chemically induced changes in surface charge or conjugation with monoclonal antibodies or integrins, modifications in substrate properties with larger molecular compounds such as matrix proteins, collagens, etc., although conducive to *in vitro* cell behavior,[25,26] would be expected to attenuate or compromise the detected OCP signal generated at the sensor interface. As with most *in vitro* cell cultures, fetal calf serum is a recognized source of cell adhesion factors, fibronectin/integrins, etc., and its presence in these experiments, particularly during seeding, enables suitable cell attachment to the gold sensors (Fig. 2).

In summary, this study using an animal model of cancer and four cytotoxic anticancer drugs has shown how an *in vitro* electrochemical assay can identify potentially "effective" drugs for subsequent *in vivo* benefit. Moreover, the technology demonstrates distinct advantages over conventional chemosensitivity assays by enabling real-time nondestructive assessments of anticancer drugs to be carried out.

Acknowledgments

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Disclosure Statement

No competing financial interests exist.

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