Electrochemical Antitumor Drug Sensitivity Test for Leukemia K562 Cells at a Carbon-Nanotube-Modified Electrode

Jing Chen,[a] Dan Du,[a] Feng Yan,[b] Huang Xian Ju,*[a] and Hong Zhen Lian[a]

Abstract: The change in electrochemical behavior of tumor cells induced by antitumor drugs was detected by using a multiwall carbon nanotubes (MWN Ts)-modified glass carbon electrode (GCE). Based on the changes observed, a simple, in vitro, electrochemical antitumor drug sensitivity test was developed. MWN Ts promoted electron transfer between the electroactive centers of cells and the electrode. Leukemia K562 cells exhibited a well-defined anodic peak of guanine at +0.823 V at 50 mV s\(^{-1}\). HPLC assay with ultraviolet detection was used to elucidate the reactant responsible for the electrochemical response of the tumor cells. The guanine content within the cytoplasm of each K562 cell was detected to be 920 amol. For the drug sensitivity tests, 5-fluorouracil (5-FU) and several clinical antitumor drugs, such as vincristine, adriamycin, and mitomycin C, were added to cell culture medium. As a result, the electrochemical responses of the K562 cells decreased significantly. The cytotoxicity curves and results obtained corresponded well with the results of MTT assays. In comparison to conventional methods, this electrochemical test is highly sensitive, accurate, inexpensive, and simple. The method proposed could be developed as a convenient means to study the sensitivity of tumor cells to antitumor drugs.

Keywords: drug sensitivity · electrochemistry · electron transfer · leukemia cells · nanotubes

Introduction

Considerable interest in the chemotherapeutic treatment of tumors can be attributed to the development of both new drugs for clinical trials and new formulations of already widely used drugs. The sensitivity of tumor cells to chemotherapeutic drugs is related to the specific pathology of the tumor and to the toxicity of the drugs in general. Thus, to select the most effective chemotherapeutic drug, a simple test is required to measure the sensitivity of tumor cells to antitumor drugs.

Antitumor drug sensitivity tests can be generally classified as either in vivo or in vitro. In vivo tests are costly, time-consuming, and complicated, which leads to difficulties in conducting clinical trials. On the other hand, in vitro tests are cost-effective, relatively rapid, and have a predictable level of activity in vivo. Consequently, a great deal of effort has been spent in establishing effective in vitro antitumor drug sensitivity tests,[1-4] such as counting by means of microscopy, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay,[1] the differential staining cytotoxicity (DiSC) assay,[2,3] and apoptosis enzyme-linked immunosorbent assay (ELISA).[4] These methods are useful in the selection of optimal therapeutic techniques and in the study of drug function. To simplify the test procedure, reduce costs, and enhance sensitivity, new drug sensitivity tests based on the electrochemical behavior of living cells, such as electron transfer at electroactive centers in cells,[5] open circuit potential at the cell/sensor interface,[6] and electric cell-substrate impedance,[7] have been developed. A drug sensitivity test involving scanning electrochemical microscopy to obtain images of the respiratory activity of collagen-embedded living cells has also been presented.[8] These studies investigated the cytotoxicity of drugs and provided alternatives to conventional drug sensitivity tests. In the present study we present a novel method of immobilizing multiwall carbon nanotubes (MWN Ts) on an electrode surface. This promotes electron transfer between electroactive centers and the electrode, thereby allowing the electrochemical behavior of living tumor cells to be monitored as a test of their sensitivity to antitumor drugs.

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Since their discovery in 1991, carbon nanotubes (CNTs) have attracted much attention due to their unique mechanical and electrical properties. CNTs have many advantages in the field of sensors: small size with larger surface area, easy immobilization of protein with the retention of activity, and in particular, the ability to facilitate electron transfer when functioning as electrode material. Consequently, this type of material has been widely used for the preparation of amperometric biosensors and in the study of the electrochemical properties of biomolecules. However, to our knowledge, no application of CNTs to cell viability studies and drug sensitivity tests has yet been reported.

This present work uses MWNTs to modify a glass carbon electrode (GCE) and proposes a novel, electrochemical method for an in vitro antitumor drug sensitivity test. The MWNT-modified GCE exhibited excellent performance in monitoring the electrochemical behavior of leukemia K562 tumor cells and in evaluating the cytotoxicity of different drugs. Leukemia K562 tumor cells showed an irreversible voltammetric response attributable to the oxidation of guanine, as shown by the results of HPLC analysis. This response was related to the action of the antitumor drugs on the tumor cells. Typical drugs with different modes of action, such as 5-fluorouracil (5-FU), mitocycin C, adriamycin, and vincristine, were selected to verify this proposed method, which combined the advantages of an electrochemical technique with those of MWNTs. The results obtained were in good agreement with those produced by MTT assay. This system provides a sensitive, accurate, inexpensive, and simple means to study the sensitivity of K562 tumor cells to antitumor drugs and should be beneficial for the development of new therapeutic drugs.

Results and Discussion

Voltammetric behavior of leukemia K562 cells: Many important processes in living cells have electrochemical characteristics. Redox reactions and changes in ionic composition derived from various cellular processes lead to electron generation and electron transfer at the interface of living cells. The cyclic voltammograms of leukemia K562 cells suspended in phosphate buffer solution (PBS, pH 7.4) at bare and MWNT-modified GCEs are shown in Figure 1. The background current of the MWNT-modified GCE (Figure 1b) was greater than that of bare GCE (Figure 1a), which might be due to the larger accessible surface area of the modified electrode. At the MWNT-modified GCE the K562 tumor cell suspension showed a well-defined anodic peak at +0.823 V at 50 mVs⁻¹ (Figure 1d), whereas no peak was observed at the bare GCE (Figure 1c). The peak current dropped dramatically after several cyclic voltammetric scans (not shown in Figure 1), and no corresponding reduction peak appeared in the inverse scan, which is characteristic of an irreversible electrode process. The typical irreversible electrochemical behavior of leukemia K562 tumor cells at the MWNT-modified electrode (Figure 1) could be attributed to the redox system of cells. By considering the irreversibility of the electrode process of K562 cells and the decrease of peak current upon continuous cyclic sweep, the peak current on the first scan could be used for the voltammetric measurement of the cytotoxicity of antitumor drugs and, therefore, as an electrochemical antitumor drug sensitivity test.

The anodic peak current increased as the amount of MWNTs on the electrode surface increased (Figure 2), indicating that the carbon nanotubes promoted electron-transfer reactions due to their structure and high electrical conductivity. At the electrode modified by the most MWNTs (10 μL of 4.0 mgmL⁻¹ MWNTs, Figure 2e) a well-defined peak was obtained and the peak current reached a maximum value. At MWNTs concentrations greater than 4.0 mgmL⁻¹, the membrane obtained was unstable and easily fell off the electrode surface.
HPLC measurements of cytoplasm: Figure 3 shows the chromatograms of guanine and a cytoplasm solution of 5 x 10⁶ K562 tumor cells per mL detected at 245 nm. The cytoplasm solution produced a UV spectrum similar to that of guanine within the range of 220 nm to 380 nm (insets Figure 3a and b). The chromatograms displayed a peak at the same retention time, indicating that the peak of the cytoplasm solution could be attributed to the cytoplasmic guanine of the K562 tumor cells. The presence of guanine was verified by liquid chromatogram-mass spectrometry (LC-MS) measurements (data not shown). To perform LC-MS analysis, the cells were fragmented under penetration pressure by suspending them in twice-distilled water for 4 h. The LC-MS spectrum of the cytoplasm solution showed a molecular ion peak of guanine at m/z 152, in which CH₃OH/H₂O (1:4 v/v) was used as a mobile phase. Moreover, the cyclic voltammograms of guanine in pH 7.4 PBS at the MWNT-modified electrode showed an irreversible oxidation peak at the potential close to that of leukemia K562 tumor cells. Thus, the anodic peak of leukemia K562 tumor cells can be attributed to the conversion of guanine to 8-oxo-guanine (Scheme 1).[24] During the electrochemical process the guanine molecules within the cytoplasm of the living cells were able to cross the cell membrane rapidly and to reach the electrode surface. MWNTs immobilized on the electrode surface acted as “molecular wire”[25] to promote the transfer of electrons between guanine and the electrode, thus enhancing the electrochemical response of the cells. These results indicate that the use of nanotechnology to amplify the electrochemical signal is suitable for studying the viability of tumor cells and the effect of antitumor drugs.

The chromatographic peak area, \( S \), of the guanine spectrum is proportional to the concentration of guanine, \( c \), within the range 0.20–2.00 \( \mu \)g/mL, with a detection limit of 0.01 \( \mu \)g/mL and a signal-to-noise ratio of 3. By using the linear regression equation [Eq. (1)], in which the relative coefficient was 0.9999, the content of guanine in the cytoplasm of each K562 tumor cell was calculated to be 920 amol:

\[
S = (5.62 \times 10^{3} c) - (1.78 \times 10^{3})
\]  

(1)

The result of HPLC detection confirmed the presence of guanine within the cytoplasm solution of K562 tumor cells. This can be attributed to the synthesis of nucleotides by salvage pathways during tumor cell growth,[23] and results in the overexpression and high activity of guanine.

Effect of 5-fluorouracil (5-FU) on the viability of tumor cells: Initially, pure biological reagent 5-FU was selected to evaluate the effect of antitumor drugs on the viability of leukemia K562 tumor cells. Upon addition of 200 \( \mu \)g/mL 5-FU to the culture medium a notable change in the voltammetric signal of the K562 cells was observed (inset in Figure 4). The presence of 5-FU caused a decrease in the peak current recorded for K562 tumor cells, indicating a reduction in the viability of these cells. This is because 5-FU is
one of the antimetabolites affecting the synthesis of nucleic acid.\textsuperscript{[26]}

As culture time increased, the peak currents of the K562 tumor cell solutions in both the presence and absence of 5-FU increased; however, the increase for the untreated cells was faster than that for the treated cells. The difference between the increasing peak currents became apparent after the cells had been cultured for 6 h (Figure 4). After 72 h the peak current of the untreated K562 tumor cells tended towards a stable value and decreased after 95 h (Figure 4a), whereas the peak current of the treated K562 tumor cells tended to a stable value after 95 h, and a decrease was not observed until 118 h had passed (Figure 4b).

The peak current is generally related to both the cell number and the expression of guanine in the cytoplasm. In the case of cells cultured in the same culture phase, the voltammetric response can show a linear relationship with cell number.\textsuperscript{[27]} As the culture time increased, the peak current of untreated leukemia K562 tumor cells did not increase linearly; rather the peak current after 118 h was lower than that after 95 h (Figure 4a). This phenomenon is due to the restricted nutrient supply during cell culture. Because the presence of 5-FU reduced cell viability, nutrient shortage occurred after a longer period than was the case for the untreated cells. Consequently, the decrease in current measured for the 5-FU-treated cells occurred after a longer period than for the untreated cells.

The dosage-dependent curve of 5-FU for a culture time of 72 h is shown in Figure 5. As the dosage of 5-FU in the culture medium increased, the peak current decreased significantly and tended towards a constant value after the concentration of 5-FU had reached 100 \( \mu g \text{ mL}^{-1} \). This change in peak current reflected the change in cell viability, which was consistent with the report by Bosanquet and Bell\textsuperscript{[29]} regarding the in vitro drug sensitivity test for chronic lymphocytic leukemia.

**Electrochemical antitumor drug sensitivity test:** The injection of three clinical antitumor drugs (adriamycin, mitocycin C, and vincristine) to the culture medium resulted in a change in the cyclic voltammetric response of tumor cells at the MWNT-modified electrode. Figure 6 shows the cytotoxicity curves for the 72 h exposure to these agents, which were applied within suitable concentration ranges, as determined by MTT assays. The antitumor mechanism of these drugs involves them binding to active target groups within the tumor cells: mitocycin C interacts with DNA\textsuperscript{[28]} and restricts its replication;\textsuperscript{[29]} adriamycin binds to DNA and induces single-stranded DNA breakage;\textsuperscript{[30,31]} and vincristine\textsuperscript{[18]} prevents protein synthesis by interacting with tubulin, the major component of the mitotic spindle.\textsuperscript{[32]} These actions affected the viability and growth of K562 tumor cells and decreased the cytoplasmic expression of guanine. The subsequent change in electrochemical response of the tumor cells enabled the cytotoxicity of the different drugs to be evaluated. All three drugs displayed cytotoxic tendencies similar to...
those of 5-FU, and in accordance with results obtained by using the conventional in vitro drug MTT sensitivity test. These observations suggest that the method of recording voltammetric measurements described above can act as a novel antitumor drug sensitivity test.

**Comparison of the proposed electrochemical antitumor drug sensitivity test with the MTT assay:** The proposed electrochemical antitumor drug sensitivity test and the MTT assay of leukemia K562 tumor cells showed increasing cytotoxicity of these three drugs as drug concentration increased (Figure 6). These curves tended towards maximum values at high drug concentrations, indicating that interactions with active target groups within the tumor cells reached saturation as the drug concentration increased; the point at which maximum cytotoxicity occurred. These curves complied with the Michaelis–Menten relationship. The development of an array incorporated with the electrochemical system, are currently under investigation.

| Drug          | Method | Cytotoxicity [%] [b] | \(c_{\text{half max}}^{[c]}\) [\%]\nmax  
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<tr>
<td>adriamycin</td>
<td>MTT</td>
<td>90.3</td>
<td>2.34</td>
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<tr>
<td></td>
<td>EC(^{[a]})</td>
<td>76.3</td>
<td>2.34</td>
</tr>
<tr>
<td>mitomycin C</td>
<td>MTT</td>
<td>72.8</td>
<td>1.09</td>
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<tr>
<td></td>
<td>EC</td>
<td>64.7</td>
<td>0.92</td>
</tr>
<tr>
<td>vincristine</td>
<td>MTT</td>
<td>64.2</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>EC</td>
<td>55.6</td>
<td>0.17</td>
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\(^{[a]}\) Percentage of maximum cytotoxicity. \(^{[b]}\) The concentration corresponding to half of the maximum cytotoxicity. \(^{[c]}\) Electrochemical method.

of these three drugs obtained by using the two assay methods were in good accordance. The slight difference might be attributed to the different culture conditions and assay procedures used. The relative errors between the results of the MTT assay and the proposed electrochemical method were acceptable, and supported the accuracy of this method and its suitability as an in vitro test for antitumor drug sensitivity.

As listed in Table 1, the maximum cytotoxicity values of the drug were not 100%, which was probably attributable to the binding equilibrium between the drugs and their target sites within the tumor cells. The drug concentration corresponding to a cytotoxicity of half of the maximum cytotoxicity was defined as \(c_{\text{half max}}\), which could be obtained from Figure 6. In fact, \(c_{\text{half max}}\) corresponds to the equilibrium dissociation constant of Michaelis–Menten kinetics, and may also be defined as the cytotoxicity constant of antitumor drugs to leukemia K562 tumor cells, which reflects the affinity of the drug to its target groups within tumor cells. Adriamycin showed a lower affinity than vincristine to the respective target groups, although it possessed a higher cytotoxicity. The \(c_{\text{half max}}\) values obtained by using the proposed electrochemical method were also very close to those obtained by using the MTT assay. Thus, the proposed electrochemical method is a credible sensitivity test for antitumor drugs.

**Conclusion**

The electrochemical antitumor drug sensitivity test combining nanotechnology offers potential advantages over conventional assays. The high accuracy is due to the electrochemical response of the electroactive substance in tumor cells following interaction with drugs. Other advantages are the possibility of signal amplification due to the involvement of MWNTs, and practicality afforded by the use of inexpensive equipment and simple procedures. By comparison, the sensitivity of the MTT assay is low, due to the relatively late decrease in mitochondrial dehydrogenase activity during the process of cell death. In addition, the apoptosis ELISA assay requires expensive reagents such as monoclonal antibodies, and the experimental procedure is laborious. The proposed electrochemical approach for an antitumor drug sensitivity test incorporating an MWNT-modified GCE is a sensitive, accurate, inexpensive and simple technique, and may be regarded as an alternative approach to study the antitumor drug sensitivity of tumor cells. The development of semi or fully automated procedures, which could be achieved by further improvements in detection equipment and the miniaturization of the three-electrode system, and the development of an array incorporated with the electrochemical system, are currently under investigation.

**Experimental Section**

**Chemicals:** 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma (St. Louis, MO, USA), 5-Fluorouracil (5-FU, Shanghai Chemical Regent Limited, China), adriamycin (Pharmacia & Upjohn Limited, China), mitomycin C (Kyowa Hakko Kogyo Limited, Japan), vincristine (Yangzhou Aasaikang Pharmaceutical Limited, China), penicillin (Huabei Pharmaceutical Limited, China), and streptomycin (Shandong Lukang Pharmaceutical Limited China) were acquired from the sources indicated. Sodium dodecyl sulfate was obtained from Huakang Science & Technology Limited, (China). High purity MWNTs (1.0 g) that had been prepared by chemical vapor deposition were boiled in 30% nitric acid at 100°C for 24 h. After separation from the mixture the sediment was washed with water until the pH reached 7.0, and then dispersed in water by performing ultrasonic agitation to give a 50 mL suspension of MWNTs. Various MWNT suspensions were prepared from this stock.

**Cell culture and collection:** Human leukemia K562 cells were obtained as a gift from the Affiliated Zhongda Hospital of Southeast University (Nanjing, China). The cells were cultured in RPMI 1640 medium (Gibco) supplemented with fetal calf serum (15%), penicillin (100 μg mL\(^{-1}\)), and streptomycin (100 μg mL\(^{-1}\)) in an incubator (5% CO\(_2\), 37°C). To study the cytotoxic effect of antitumor drugs on cell viability, various drugs were added to other formulations of cell culture medium. The same culture conditions were provided for both the control and experimental groups. The cells for different groups were cultured from the same initial number of cells (2.5×10⁴ mL\(^{-1}\)) in flasks of the same size. The cells were counted by using a Petroff–Hauser counter (USA).

The cultured leukemia K562 cells were separated from the culture medium by performing centrifugation at 1000 g for 10 min, and were...
then washed twice with sterile PBS (pH 7.4) containing NaCl (137 mM), KCl (2.7 mM), NaHPO₄ (87 mM), and KH₂PO₄ (14 mM). The sediments were suspended in PBS to give cell suspensions of 1.0 mL for use in electrochemical measurements.

**Production of the MWNT-modified GCE:** Prior to modification, glassy carbon electrodes (GCE; 3 mm in diameter) were polished successively with 1.0 µm, 0.3 µm, and 0.05 µm alumina slurries, followed by rinsing thoroughly with doubly distilled water. After successive sonication in a 1:1 mixture of nitric acid and doubly distilled water, the electrodes were dried at room temperature in a desiccator.

After the MWNTs suspension was stirred to allow the MWNTs to fully disperse, a quantity (10 mL, 4.0 mgmL⁻¹) was cast onto the surface of the GCE. After varying amounts of MWNTs suspensions were added in a drop-wise manner until an optimal coating was achieved. The electrodes were dried in a desiccator to give the MWNT-modified GCEs. These were then electrochemically treated by performing several cycles of between 0.0 and +1.1 V in pH 7.4 PBS until stable background lines were obtained.

**Electrochemical measurements:** Electrochemical experiments were performed using a CHI 730 electrochemical analyzer (USA) with a conventional three-electrode system comprising a platinum wire as auxiliary, a saturated calomel electrode as reference, and a MWNT-modified GCE as the working electrode. The electrochemical behavior of leukemia K562 cells was studied by using pH 7.4 PBS and cyclic voltammetry in the potential range from 0.0 to +1.1 V with a scan rate of 50 mVs⁻¹ at room temperature. The cytotoxicity of the drug was calculated as follows:

\[
\text{Cytotoxicity} (\%) = \frac{|I_{ctrl} - I_{drug}|}{I_{ctrl}} \times 100
\] (2)

in which \(I_{ctrl}\) is the peak current of the cells in the absence of antitumor drug treatment and \(I_{drug}\) is the peak current of the cells treated with drugs.

**MTT assay:** Leukemia K562 cells (1×10⁵) in 200 µL of either medium alone or medium containing drugs at various concentrations were added to each well of a 96-well plate. The plate was incubated at 37°C in a humidified atmosphere of 5% CO₂ for three days. MTT (20 µL, 5 mgmL⁻¹) was then added to each well. After the plate was incubated for a further 4 h, sodium dodecyl sulfate (150 µL, 0.520%) was added to each well to solubilize the formazan dye. After 1 h the absorbance of the control and drug-treated wells was measured using a Wallac Victor1420 Multilabel Counter at 490 nm. The cytotoxicity of the drug was calculated as follows:

\[
\text{Cytotoxicity} (\%) = 1 - \frac{\text{absorbance of drug-treated well}}{\text{absorbance of control well}} \times 100
\] (3)

**HPLC measurement:** Leukemia K562 tumor cells (5×10⁴) were incubated in a lysis buffer at 4°C for 10 min. The pH of the lysis buffer (Tris (20 mM), KH₂PO₄ (3 mM), NaCl (120 mM), MgCl₂ (1.0 mM), CaCl₂ (1.2 mM), and glucose (10 mM)) was adjusted to pH 7.4 by addition of HCl. This mixture was then centrifuged at 1000 g for 10 min to remove cellular cytoskeleton and nuclei. The supernatant was further centrifuged at 15000 g for 30 min and pH 7.4 PBS was added to give a sample of cytoplasm (1.0 mL), which was subjected to HPLC analysis.

HPLC detections were performed at 245 nm with a Varian 5060 separations module (Waters Milford, MA, USA). The chromatographic separation was performed with a Kromasil ODS column (4.6 mm×150 mm) at 30°C equipped with a Waters 486 tunable absorbance detection system. The mobile phase, consisting of 5% CH₃OH and 95% H₂O, was run at 1.0 mLmin⁻¹. 10 µL of each sample was injected into the HPLC system for analysis.

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