Analytical Methods

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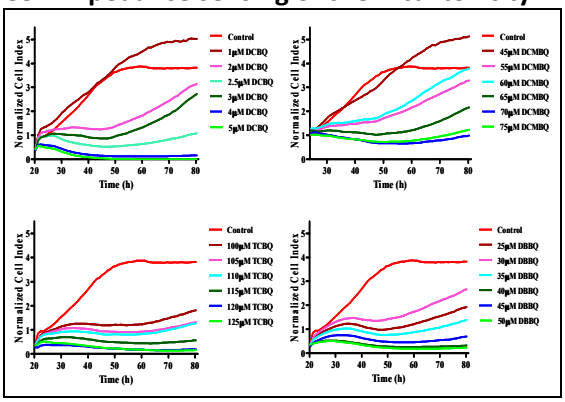
Graphic content

Cell-impedance sensing of chemical toxicity



A real-time cell-electronic sensing method for comparative analysis of toxicity of water contaminants

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Abstract:

We report a real-time cell-electronic sensing (RT-CES) technique using T24 bladder cancer cell line as the probe to analyze comparative cytotoxicity of water contaminants. The RT-CES system consists of a 96-well E-plate embedded with microelectrodes on each micro-well. As T24 cells grow on the surface of the microelectrodes, the impedance between the cell-microelectrodes continuously increases with the increase of cell numbers. When the cells are exposed to a chemical, the toxic effects on the cells resulting in cell death, detachment, and morphological changes reduce impedance. These dynamic changes are continuously monitored to provide real-time detection of cell responses. The application of this method was successfully used to analyze cytotoxic characteristics of four halobenzoquinones (HBQs), emerging disinfection byproducts in drinking water, including 2,6-dichloro-1,4-benzoquinone (DCBQ), 2,6-dichloro-3-methyl-1,4-benzoquinone (DCMBQ), 2,3,6-trichloro-1,4-benzoquinone (TCBQ), and 2,6-dibromobenzoquinone (DBBQ). T24 cells were seeded in each of the 96 microwells on the 96-E-plate, and treated with varying concentrations of the four HBQs. The RT-CES technique provides continuous profile of HBQ induced cytotoxicity response curves and temporal IC_{50} histograms in T24 cells, demonstrating concentration-, time- and compound- dependent

cytotoxic effects. These results enable ranking of cytotoxicity of the four HBQs: DCBQ＞DBBQ ＞DCMBQ＞TCBQ. We further examined and compared cytotoxicity of HBQs using the conventional assays, including the Neutral Red Uptake (NRU) and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium(MTS) assays, to complement and correlate with the RT-CES data. The 24 h IC_{50} value of HBQs obtained from RT-CES was correlate with NRU assay (R^2=0.9480, P<0.05), while MTS results presented moderate and less consistent correlation with the results of the RT-CES and NRU (R^2=0.5281 and 0.3144, respectively). All three assays show that DCBQ cause greater toxic effects in T24 than the other three HBQs, although the IC_{50} values are dependent on the assays. The RT-CES technique can provide sensitive and high throughput testing of cytotoxicity of environmental contaminants.

Introduction

Cell-based electrochemical biosensors have become powerful tools for assessment of potential risks of environmental contaminants to ecological systems and/or human health. Among the different types of cell-based electrochemical biosensors, impedance-based ones are particularly interesting because they can provide real-time, label-free and more sensitive detection of cellular changes when exposed to the testing substances.[1] The real-time cell-microelectronic sensing (RT-CES) technique is one of such examples. The RT-CES system consists of a 96-well E-plate embedded with microelectrodes on each micro-well. Live cells can grow on the surface of the microelectrodes. As the cell population and attachment increase, impedance on the cell-microelectrodes continuously increases, which is continuously monitored to provide real-time sensing.[2, 3] In recent years, the RT-CES technique has been applied to monitor cytotoxicity of chemicals[2-5] and screen of drugs.[6, 7]

Disinfection byproducts (DBPs) arise from the reactions of common disinfectants with natural organic matter in the source water.[8] The presence of DBPs in treated drinking water is important to manage potential risk on public health, because several epidemiological studies have found a potential association between long term exposure to disinfected water and increased risk of human bladder cancer and developmental toxicity.[9-12] Although over 600 DBPs have been identified, toxicological evaluation of the identified DBPs has only been conducted for a small number of DBPs.[8] Therefore it is in urgent to develop a more sensitive and rapid method to assess and screen the toxicity of DBPs. We have previously identified four halobenzoquinones (HBQs) as new DBPs in chlorinated and chloraminated water.[13, 14] These include 2,6-dichloro-

1,4-benzoquinone (DCBQ), 2,6-dichloro-3-methyl-1,4-benzoquinone (DCMBQ), 2,3,6-trichloro-1,4-benzoquinone (TCBQ), and 2,6-dibromobenzoquinone (DBBQ). These HBQs were also present in swimming pools, and the concentration is much higher than that in tap water.[15] DCMBQ and TCBQ were predicted to be carcinogens based on quantitative structure toxicity relationship (QSTR) analysis.[16] In addition, these four HBQs are cytotoxic to human bladder cancer cells and cause oxidative damage to DNA and protein.[17] Therefore, these four HBQs were chosen as the target chemicals for developing a RT-CES method to further assess their cytotoxicity.

The objective of this study is to develop a RT-CES method for assessment and comparison of the cytotoxicity of HBQs. Human bladder epithelial cancer cell line T24 was chosen as the sensing probe to examine time- and dose- dependent cytotoxic responses to HBQs, because T24 was previously shown to be sensitive to environmental contaminants and it is relevant to the potential risk of bladder cancer.[2,3,9,11] We further examined and compared cytotoxicity of HBQs using the conventional assays, including the Neutral Red Uptake (NRU) and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium(MTS) assays, to complement and correlate with the RT-CES data.

Experimental

Reagents

Standards of DCBQ, DCMBQ, TCBQ and DBBQ were purchased from Sigma–Aldrich, Shanghai Acana Pharmtech, INDOFINE Chemical Company and Fluka (Table 1). HBQ solutions were separately prepared in methanol (HPLC grade, Fisher Scientific). McCoy's 5A Modified Medium with L-Glutamine was purchased from American Type Culture Collection

(ATCC). 1×Dulbecco's Phosphate-Buffered Saline (DPBS), 0.05% Trypsin-EDTA and penicillin/streptomycin (P/S) were purchased from Gibco. Fetal bovine serum (FBS) and Neutral Red solution were purchased from Sigma–Aldrich.

Cell culture

Human bladder epithelial cancer cell line T24 was obtained from ATCC (Manassas, VA) and incubated in the medium containing McCoy's 5A modified medium with 10% FBS and 1% P/S at 37 ℃ with 5.0% CO_2. Cells were treated in the logarithmic growth phase.

RT-CES testing of cytotoxicity

The procedures of the RT-CES 96×system (ACEA Biosciences) for cytotoxicity testing have been described elsewhere.[3, 5] This system is composed of E-plate station, 96-well E-plate and the system analyzer. RT-CES measures "cell index" (CI), a unitless measurement of cell morphology, viability, and proliferation, in real time.[18, 19] CI corresponds to the impedance caused by cells adhering to microelectrodes on the bottom of specialized microwell plates; this impedance (reported as CI) is continuously measured by the RT-CES system. If CI increases, this may indicate combined effects of cell proliferation, growth, and/or increased cell surface area in contact with the microelectrodes; if CI decreases, this may indicate lack of proliferation, cell death, detachment and/or decreased cell-electrode contact.

An initial number of T24 cells were seeded in each of the 96 microwells on the 96-E-plate. The cells were allowed to grow for approximately 20 h when the CI reached 1 unit. The cells on each of the microwells were treated with one of the four HBQs at a given concentration. This was completed by replacing the culture media with the testing media containing different concentrations of one of the HBQs (200 μL/well). The concentrations tested were DCBQ (1-5

μM), DCMBQ (45-75 μM), TCBQ (100-125 μM), and DBBQ (25-50 μM). Negative controls were included, where the cells grew in the normal media without any of the HBQs. The solvent controls were also included, where the cells grew in the normal media containing 1.33% v/v methanol, the largest amount of methanol used to test HBQs. On each 96-E-plate, both negative and solvent controls were included. Triplicate tests were used for each concentration. The cultures were continuously monitored for up to 80 h. The experiments were repeated for another two times. Prism 5 software (Graphpad Software) was used for data analysis. The cell index (CI) data were analyzed to calculate dose-response curves. Cell viability is defined as the relative CI of the treated cells to that of the control cells at a given time.

Neutral Red Uptake (NRU) assay

The NRU assay is based on the ability of viable cells to incorporate and bind Neutral Red within lysosomes.[20] T24 cells were treated with one of the four HBQs with varying concentrations: DCBQ (5-75 μM), DCMBQ (75-200 μM), TCBQ (75-175 μM), and DBBQ (25-150 μM). After the cells were treated for 24 h, an aliquot of 20μL of 1% Neutral Red solution was added to each well, and incubated at 37 °C for 2 h. The cells were then washed with PBS, and fixed with 50 μL of solubilization solution (1% acetic acid in 50% ethanol) in each well. The plate was immediately placed in a Microplate Spectrometer (Bio-Rad, Benchmark Plus), shaken for 10 minutes, and absorbance for each well was measured at a wavelength of 540 nm with a reference wavelength of 690 nm.

Data analysis

The IC_{50} represents a concentration causing 50% reduction in viability.

Statistical analysis was performed using GraphPad Prism 5 and OriginPro 8.5 (OriginLab Corporation). Experimental results were expressed as mean ± standard deviation (SD). One way analysis of variance (ANOVA, followed by Dunnett's Post Hoc Multiple Comparisons) was used for multiple comparisons between treatment and control groups for all tests. Comparison of different assays was made by correlation and linear regression analysis. Difference was considered to be statistically significant at $P<0.05$.

Results and discussion

RT-CES method development

We first optimized the proper cell growth of T24 cells in the E-plate. An initial population of 3000-7000 cells per well were seeded in the 96-E-plate. Figure 1A shows the cell index over time, indicating the cell growth curve of T24 cells. At the time zero, the cell index is zero because no cells were attached to the microelectrodes. With the time increases and cells proliferate, the cell index increases because the number of cells attached to microelectrodes is continuously increasing. Figure 1A also shows that the number of the initial cells seeded is linearly correlated with the cell index. Figure 1B shows the linear relationship between the cell index at 24 h and the cell number we seeded ($R^2=0.9921$, $P<0.01$). Based on Figure 1, the number of T24 cells for seeding was chosen as 4000 for the following experiments. Because HBQs need methanol to dissolve, we also included 1.33% methanol control (the highest concentration we may use). Figure 1C shows a typical RT-CES profile of T24 cells exposed to solvent control, 1.33% methanol (v/v) in culture medium. No statistically significant difference of CI value at 24 h was observed between negative control and methanol control (student t-test).

RT-CES continuous profiling of HBQ induced cytotoxicity in T24 cells

Having established the RT-CES method for monitoring T24 cells, we investigated the responses of T24 cells to HBQ exposure over time, temporal profiling of cytotoxicity of HBQs. T24 cells were exposed to varying concentrations of HBQs and monitored for up to 80 h. Figure 2 shows the RT-CES continuous monitoring of the profiles of T24 cells responding to the treatments with the four HBQs. The RT-CES acquired the CI from each microwell at a 1 h interval during the entire experimental time. The control groups (T24 cells without HBQ treatment) showed normal cell growth. When T24 cells were treated with any of the four HBQs, the CI decreased with increasing concentrations of HBQs. This reduction in CI is corresponding to combined effects of cell death, cell detachment from the microelectrodes, and/or inhibition of proliferation due to HBQ exposure. At the highest concentration tested, each of the four HBQs reduced CI to near zero, indicating few or no viable cells were attached to the microelectrodes. The maximum cytotoxic effect was observed at 5 μM for DCBQ, 50 μM for DBBQ, 75 μM for DCMBQ, and 125 μM for TCBQ. These concentrations were statistically different (P<0.05). These results suggested that DCBQ is the most toxic to T24 cells amongst the four HBQs. In support of this, temporal IC_{50} histograms over 75-hours of treatment were obtained from the continuous profiling data, as shown in Figure 3. This clearly shows that the cytotoxicity of the four HBQs is in the order of DCBQ＞DBBQ＞DCMBQ＞TCBQ.

In addition to the IC_{50} values and the order of toxicity, the RT-CES continuous profiles (Figure 2 and Figure 3) can provide more information about dynamic cell responses to the treatment of HBQs. The sensing profiles show two phases of the cell responses. During the first 30 h after HBQ treatment (phase 1), the normalized CI values continue to decrease, which is due to the toxic effects induced by HBQs. After 30 h exposure to HBQs (phase 2), the normalized CI

values begin to increase over time, indicating that T24 cells may be able to recover from the cytotoxic effects of HBQ exposure. The recovery of T24 cells exposed to HBQs may be due to the metabolism of HBQs in cells, indicating that the transformed compounds of HBQs may less toxic; and/or T24 cells have repair capability to remove damages and to recover from some toxic effects. The ability of showing dynamic changes is a unique advantage of the RT-CES method over the traditional assays.

NRU assay of viability of T24 cells after HBQ treatments

The NRU assay was performed for two purposes: (1) confirming the cytotoxic effects of HBQs on T24 cells and (2) comparing IC_{50} values with those from the RT-CES testing. Figure 4 presents the viability curves of T24 cells obtained using NRU when the cells were treated for 24 h with any one of the four HBQs with varying concentrations. The HBQ treatment groups showed significantly lower viability compared with the control groups at all concentrations (one-way ANOVA analysis, $P<0.05$). The IC_{50} values measured with the NRU assay were 11 μM (DCBQ), 147 μM (DCMBQ), 113 μM (TCBQ) and 45 μM (DBBQ), demonstrating the cytotoxic effects on T24 in the order of DCBQ>DBBQ>TCBQ>DCMBQ. Both NRU and RT-CES results support the hypothesis that HBQs cause cytotoxicity, and DCBQ and DBBQ are more cytotoxic than DCMBQ and TCBQ to T24 cells.

Correlation between RT-CES, NRU and MTS assay

We further compared the IC_{50} values of the 4 HBQs determined with the RT-CES assay with those determined with the NRU assay. In addition, we compared RT-CES data with the IC_{50} data obtained using MTS assay that has been reported in the previous study.[17] Figure 5 showed linear correlation of the IC_{50} values from the RT-CES with those obtained with NRU assays for the

four HBQ compounds (linear correlation coefficient of R^2=0.9480, P<0.05), while MTS results presented moderate and less consistent correlation with the results of RT-CES and NRU (R^2=0.5281, R^2=0.3144). The correlation between the RT-CES and NRU assays is consistent with the previous studies.[5, 21] The NRU assay is one of the recommended assays by the National Institutes of Health (NIH) and the National Institute of Environmental Health Sciences (NIEHS) for determining the cytotoxicity of unknown compounds when used with the NIH:3T3 cell line.[22] Our results indicate that the RT-CES assay and NRU results are generally consistent.

In this study, we have developed and affirmed that RT-CES technique can be used to assess cytotoxicity of DBPs, showing the promise of this technique for high-throughput, and more sensitive testing of environmental contaminants. Real-time monitoring can provide continuous data of cytotoxicity. We have studied cytotoxicity of the four HBQs with a bladder cancer cell line T24 because this cell line has shown high sensitivity to other DBPs such as nitrosamines.[2] Another reason for using T24 cell line as the sensing probe is that increased risk of human bladder cancer is the major adverse effects of DBPs according to the epidemiological studies.[9, 11] The RT-CES technique provides a continuous, rapid, and independent monitoring of cytotoxicity on 96 wells, allowing determination of an IC_{50} histogram. For example, after 24, 48, and 72 h of exposure, IC_{50} values of DCBQ are 1.9, 2.2, and 3.1 μM, respectively, which can be compared with other assays. Being widely used in *in vitro* toxicology studies, NRU and MTS assays were also conducted to confirm the toxicity of HBQs. All three assays show that DCBQ cause greater toxic effects on T24 than the other three HBQs, although the IC_{50} values are dependent on the assays. The difference among different assay is likely due to the different principles of detection used in the three assays. The NRU assay is based on the ability of viable cells to incorporate and

retain Neutral Red dye, which is dependent on ATP production and maintenance of pH gradients.[20] The MTS assay revealed a reduction in the mitochondrial dehydrogenase activity and is directly proportional to the number of living cells.[23] The IC_{50} values at 24 h obtained by RT-CES are lower than those by the other two methods. However, as assessed by pairwise correlation of IC_{50} values, the NRU results are consistent with those obtained by RT-CES (R^2=0.95), while MTS results present a moderate and less consistent correlation with the results of RT-CES and NRU (R^2=0.53, R^2=0.31). Furthermore, the various profiles of cytotoxicity that are detected by different methods may indicate the involvement of different cytotoxic mechanisms of these HBQs.

Conclusion

Taken together, this study demonstrates that the RT-CES technique is able to more sensitively detect the cytotoxicity of HBQs and provides richer information (continuous toxicity profiles, IC_{50} histogram, and dynamic cellular responses to toxicants) compared with NRU and MTS assays (frequently used endpoints data). The results from all three assays clearly show the differences in the cytotoxicity of the four HBQs, and that DCBQ causes the greatest toxic effects on T24 cells. The cellular injury may lead to complex changes to structural changes and molecular events, such as the plasma membrane, mitochondria, lysosomes, et al, resulting in cell death. In summary, RT-CES technique can be useful and promising in application of assessing cytotoxicity of environmental contaminants, and provides firsthand information about cytotoxic effects to direct future *in vivo* toxicological studies.

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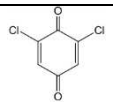
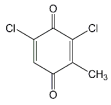
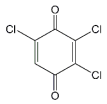
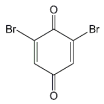
Table and Figures

Table 1 Characteristics of the halobenzoquinones analysed in this study

Halo-benzoquinones	CAS #	Molecular Formula	Source and Purity	Structure
2,6-dichloro-1,4-benzoquinone, DCBQ	697-91-6	$C_6H_2Cl_2O_2$	ALDRICH, 98%	
2,6-dichloro-3-methyl-1,4-benzoquinone, DCMBQ	40100-98-9	$C_7H_4Cl_3O_2$	ALDRICH, 98%	
2,3,6-trichloro-1,4-benzoquinone, TCBQ	634-85-5	$C_6HCl_3O_2$	Shanghai Acana Pharmtech Co., ≥98%	
2,6-dibromo-1,4-benzoquinone, DBBQ	19643-45-9	$C_6H_2Br_2O_2$	INDOFINE Chemical Company, ≥98%	

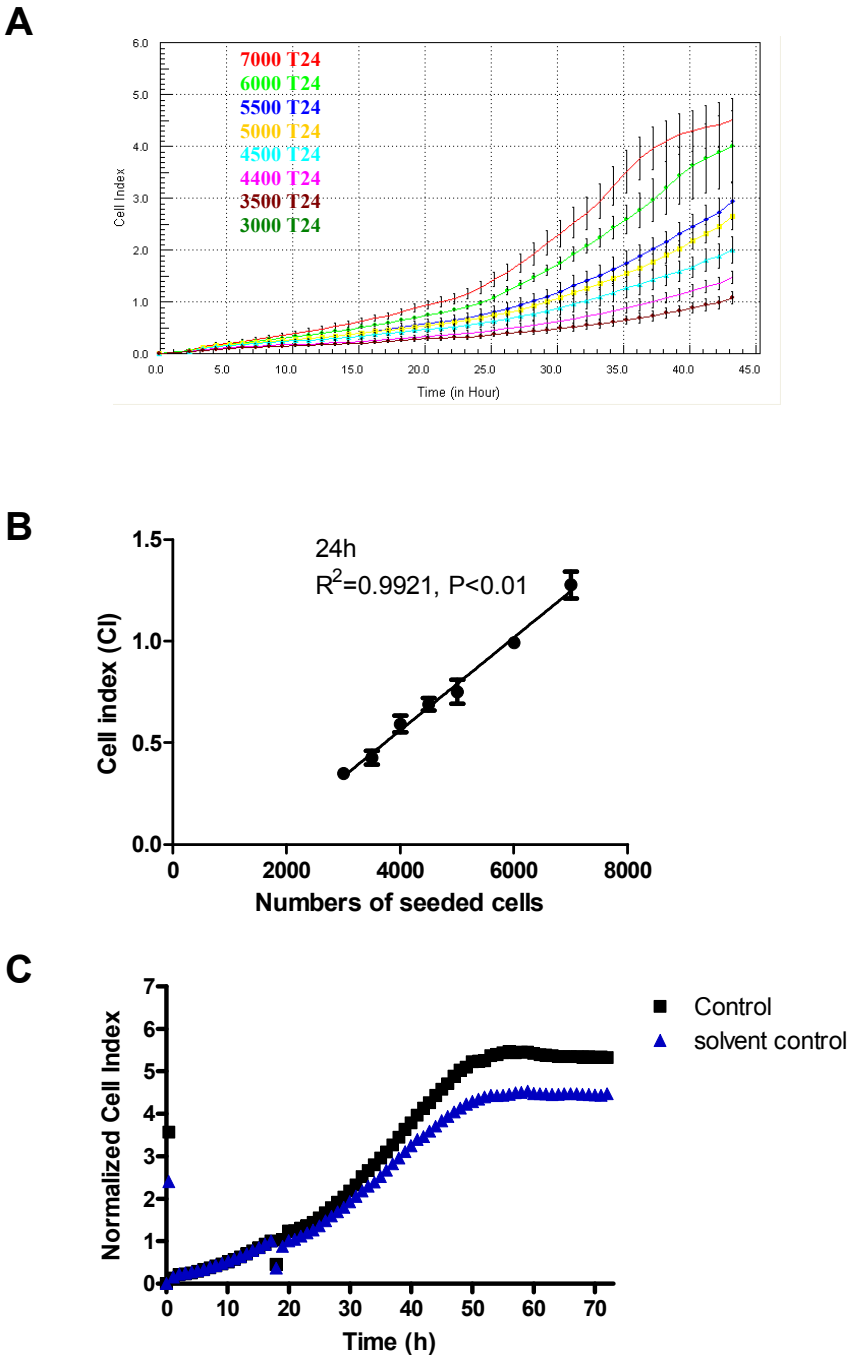


Figure 1 Method development with RT-CES system. (A) The growth curves of T24 cells at varying seeding numbers. (B) The linear relationship between cell index and the numbers of seeding cells at 24 h after seeding. (C) RT-CES profiles of T24 cells in normal culture media (control) and in the media containing 1.33% MeOH (solvent control).

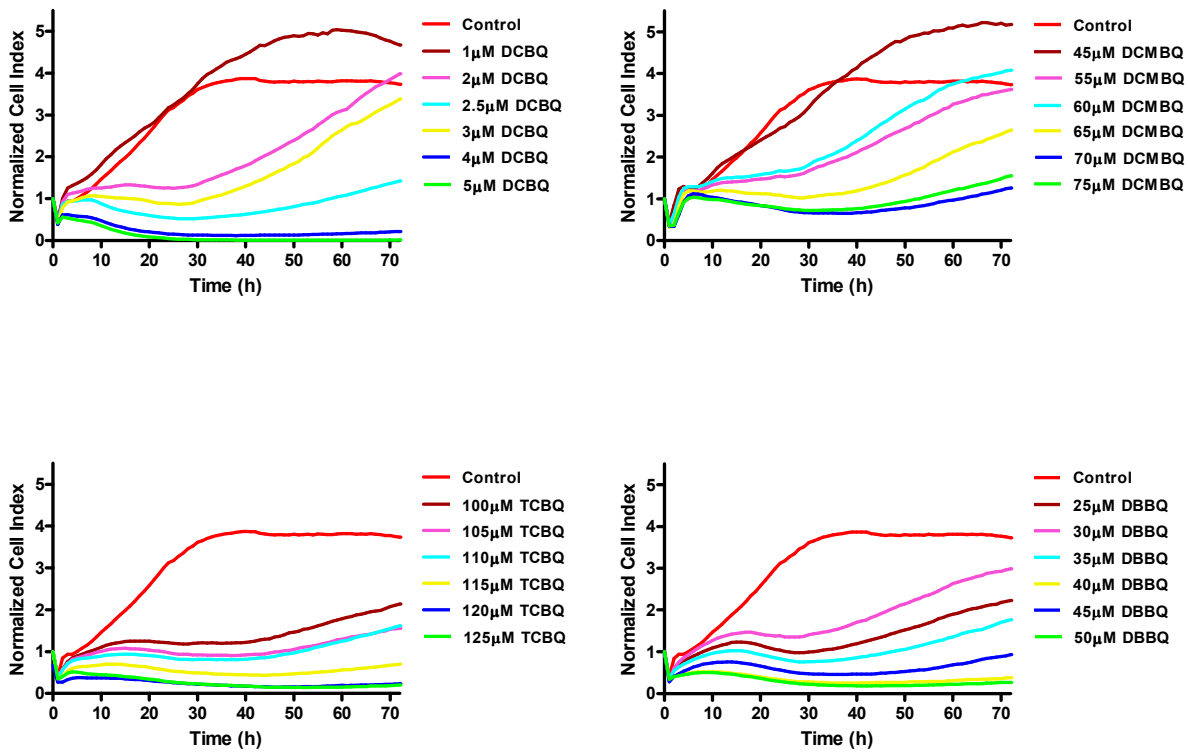


Figure 2 RT-CES dynamic responses curves (CI values over exposure time) showing the effect of four HBQs on T24 cells. T24 cells were seeded into the 96 × E-plate and cell proliferation was monitored every hour. Once the cells reached the exponential growth phase, approximately 20 h later when the cell index increased to 1, they were treated with different concentrations of HBQs. Each trace at each concentration was an average of three replicates.

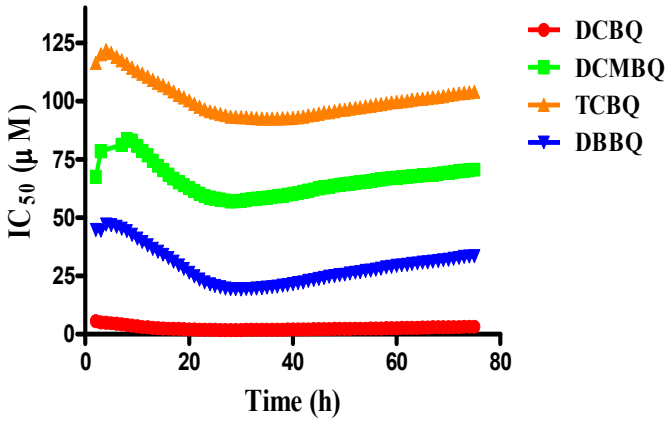


Figure 3 IC_{50} histograms over 75 h post-treatment of HBQs. The IC_{50} values were calculated and displayed at each exposure time points. Using RT-CES, the IC_{50} was obtained to be 1.9 μM for DCBQ, 58.7 μM for DCMBQ, 95.6 μM for TCBQ and 21.4 μM for DBBQ after 24 h exposure.

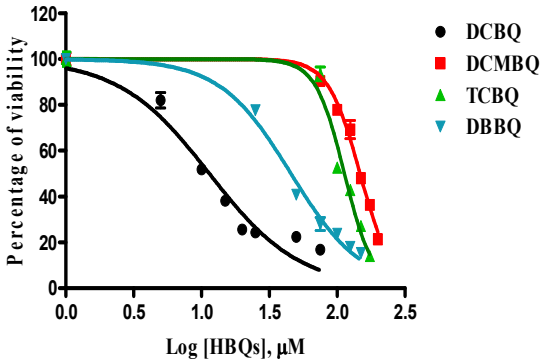


Figure 4 Neutral red uptake measurements of cell viability curves of T24 cells treated with DCBQ, DCMBQ, TCBQ, or DBBQ for 24 h. All values are expressed as mean ± SD. The significance level observed was $P < 0.05$ in multiple comparisons.

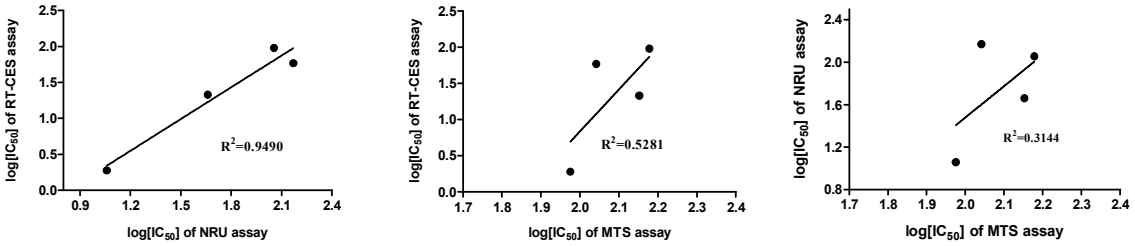


Figure 5 Pairwise correlation of IC_{50} among RT-CES, NRU and MTS cytotoxicity assays. The linear relationships between two assays were found. IC_{50} obtained by NRU assay seemed to be consistent with those obtained by RT-CES (R^2=0.9490, P<0.05), while MTS results presented moderate and less consistent correlation with the results of RT-CES and NRU (R^2=0.5281, R^2=0.3144)